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Bajpai et al., "Effects of Aging Mortierella Mycelium on Production of Arachidonic and Eicosapentaenoic Acids", pp. 775-780, 1991, JAOCS, vol. 68, Oct.

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T hni al

Production of Eicosapentaenoic Acid by *Mortierella* Fungi

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My elia of arachidonic acid-producing fungi belonging to the genus *Mortierella* were found to be rich sources of 5,8,11,14,17-*cis*-eicosapentaenoic acid (EPA). Production of EPA by these fungi was observed only when they were grown at low temperature (6–16 C). EPA comprised 5–20% of the total extractable mycelial fatty acids in most strains tested. No significant accumulation of EPA was observed on incubation at high temperature (20–28 C), at which the other major mycelial C-20 fatty acid, arachidonic acid, was still efficiently produced. In a study on the optimization of the culture conditions for EPA production by a selected fungi *M. alpina* 20–17, a medium containing glucose and yeast xtract as major carbon and nitrogen sources, respectively, was found to be suitable. Periodic feeding of glucose during growth of the fungus and cultivation at high temperature (20 C) during the early growth phase followed by temperature shift to 12 C were found t be effective at increasing mycelial yield and reducing cultural period, respectively. Under the optimal culture conditions, the EPA production reached 0.49 mg/ml of culture broth (29 mg/g dry mycelia). This value accounted for 13.5% of the total fatty acids in the extracted lipids. Other major fatty acids in the lipids were palmitic acid (6.0%, by weight), stearic acid (5.3), oleic acid (6.2), linoleic acid (3.0), γ-linolenic acid (3.5) and arachidonic acid (60.0).

5,8,11,14,17-*cis*-Eicosapantaenoic acid (EPA) is a rare C-20 polyunsaturated fatty acid of potential pharmaceutical value. This fatty acid has been shown to be effective in preventing blood platelet aggregation (1). It has also been demonstrated to be useful for blood cholesterol reduction, thus reducing the risk of atherosclerosis (2). Several marine fish oil products have become available recently as lipid sources relatively rich in EPA. For practical purposes, however, these conventional sources are not satisfactory, due to their low EPA contents and the presence of other fatty acids with less desirable properties. A marine alga, *Chlorella minutissima* (3), a freshwater alga, *Monodus subterraneus* (4), a moss, *Lepotobryum pyriforme* (5), and *Euglena gracilis* (6), have been suggested as alternative sources of EPA. But, again, they are not so advantageous because of their poor growth rates and low lipid contents. To obtain more suitable sources for large-scale preparation of EPA, we have started screening lower classes of microorganisms, i.e., bacteria and fungi, as to the ability to accumulate lipids containing EPA. No attention has been paid so far to such microorganisms as sources of EPA.

In previous papers (7,8), we reported that several fungi accumulate large amounts of arachidonic acid when grown in usual media containing glucose as a major carbon source. We have now found that these arachidonic acid producers, especialy strains belonging to the genus *Mortierella* subgenus *Mortierella*, specifically accumulate EPA in their mycelia when grown at low temperature (9). In this work, the potential of various arachidonic acid-producing fungi as sources of EPA, and the environmental and nutritional conditions under which maximum EPA productivity can be obtained, were studied.

MATERIALS AND METHODS

Chemicals. Fatty acid methyl esters were purchased from Funakoshi Chemicals, Tokyo. All other reagents used in this work were of analytical grade and commercially available.

Microorganisms, media and cultivations. All fungal strains used were from our stock cultures (AKU Culture Collection, Faculty of Agriculture, Kyoto University). Medium GY contained 2% glucose and 1.0% yeast extract, pH 6.0. Medium YM was described previously (7). Each fungus was inoculated into 50-ml shaking flasks containing 10 ml of either medium GY or YM and then incubated at 12 C for seven days with reciprocal shaking (120 strokes/min), unless otherwise stated.

Extraction and determination of fatty acids. Fungal cells were harvested by suction filtration, washed with 50 ml of water and then dried at 100 C overnight. The dried cells were suspended in five ml of methylene chloride–10% methanolic HCl (1:1, v/v) for three hr at 50 C. As an internal standard, *n*-heptadecanoic acid (0.5 mg) usually was included in the methanolysis mixture. After extraction with 20 ml of *n*-hexane, followed by evaporation, the fatty acid methyl esters were dissolved in 0.05–0.1 ml of acetonitrile and then analyzed by gas liquid chromatography (GLC). The conditions for GLC were the same as those described previously (7) except for the following changes: glass column (3 mm × 2 m) packed with 5% Advans DS on 80/100 mesh Chromosorb W (Shimadzu, Kyoto); column temperature, 190 C, and injection port temperature, 240 C. Mycelial fatty acid composition values are given in weight percent.

Isolation of the EPA methyl ester from fungal mycelia. The mycelia from two separate cultures f *Mortierella alpina* 20–17, each f which was grown in 50 ml of medium GY in a 500-ml flask under the optimal conditi ns as described below, were collected by suction filtrati n and then washed with 200 ml of water. The procedures used for transmethylation and purifi-

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TABLE 1

Comparison of EPA Productivities and Changes in Mycelial Fatty Acid Composition in *Mortierella* Fungi Grown at 12 and 28 or 24 C[a]

Strain	Temperature (C)/ cultivation period (days)/medium	Productivity: Mycelial mass (mg/ml of culture medium)	Productivity: Total FA[c] (mg/g dry mycelia)	Productivity: EPA content (mg/g dry mycelia)	Productivity: EPA yield (μg/ml of culture medium)	Fatty acid composition (%)[b]: 16:0	18:0	18:1	18:2	18:3	20:3	20:4	20:5	others
M. hygrophila	12/7/GY[d]	5.5	94	9.8	54	16.3	3.8	30.7	8.9	11.3	2.5	13.6	10.4	2.5
IFO 5941	28/6/GY[d]	7.0	183	0	0	24.7	2.9	37.4	9.5	5.5	1.6	17.9	0	0.5
M. zychae	12/10/GY	10.0	166	4.4	44	15.5	15.1	31.9	6.4	5.0	5.9	15.0	2.7	2.5
CBS 652.68	28/7/GY	7.7	120	0	0	23.2	12.6	29.3	8.7	6.0	3.3	16.3	0	0.6
M. elongata	12/10/GY	10.0	189	9.6	95	14.3	15.4	35.8	5.1	4.4	3.8	12.6	5.0	3.6
CBS 121.71	28/7/GY	8.1	61	0	0	15.4	14.0	30.3	6.7	6.0	3.8	21.7	0	2.1
M. elongata	12/7/YM	5.8	76	6.0	35	15.5	4.2	31.6	8.9	7.6	2.9	15.5	7.9	5.9
1S-5 AKU 3999	24/6/YM	6.9	166	0	0	14.5	8.0	34.0	7.6	6.0	2.6	23.6	0	3.7
M. parvispora	12/7/YM	3.0	77	8.4	25	8.8	2.2	24.5	15.3	13.8	2.4	14.4	10.9	7.7
2S-13 AKU 3994	28/6/YM	8.0	201	0	0	7.8	9.0	56.3	5.3	6.9	1.9	9.8	0	0
M. schmuckeri	12/7/GY	10.4	219	4.9	51	24.6	11.6	39.1	5.3	3.2	4.0	5.8	2.3	4.1
NRRL 2761	28/5/GY	8.0	205	0	0	19.9	12.4	37.1	7.4	4.9	4.9	12.4	0	1.0
M. alpina 1S-4	12/7/GY	3.5	59	8.1	28	8.7	2.2	16.8	14.4	10.1	3.0	28.4	13.9	2.9
AKU 3998	28/6/GY	9.5	318	0	0	17.9	5.9	11.3	9.8	4.1	3.3	47.7	0	0
M. alpina 2O-17	12/7/GY	4.6	71	12.1	55	7.6	1.2	9.2	11.0	11.2	3.6	38.7	17.1	1.7
AKU 3996	28/6/GY	9.4	277	0	0	15.8	5.3	12.0	18.2	4.8	2.3	39.6	0	0.7
M. alpina	12/10/GY	8.8	109	6.8	60	13.6	5.4	24.2	11.4	7.2	5.4	21.0	6.2	5.6
CBS 250.53	28/7/GY	6.2	124	0	0	14.5	5.9	27.8	11.4	7.4	4.0	27.1	0	1.9
M. alpina 1-83	12/7/GY	3.8	58	11.6	44	9.2	1.3	15.7	14.1	11.4	1.8	24.2	19.8	2.5
AKU 3995	28/6/GY	9.4	300	0	0	18.6	4.8	12.3	8.9	4.1	3.4	47.9	0	0
M. alpina	12/10/GY	9.7	187	7.1	69	16.9	12.0	17.8	7.4	6.6	10.1	22.3	3.8	3.1
CBS 219.35	28/7/GY	5.5	139	0	0	11.2	4.9	30.5	14.4	10.9	4.1	22.4	0	1.7

[a]Each strain was grown under conditions described in the text except for growth temperature and cultivation period as indicated.
[b]16:0, palmitic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, γ-linolenic acid; 20:3, dihomo-γ-linolenic acid; 20:4, arachidonic acid; 20:5, EPA. α-Linolenic acid and other polyunsaturated fatty acids of the n-3 series were not detected.
[c]FA, fatty acid.
[d]Medium GY and medium YM contained 2% glucose and 1% yeast extract (pH 6.0), and 1% glucose, 0.5% peptone, 0.3% yeast extract and 0.3% malt extract (pH 5.0), respectively.

cation of EPA were essentially the same as described previously (7).

Other methods. Fungal growth was measured by determining the mycelial weight after drying at 100 C overnight. Glucose concentrations in media were measured with a commercially available kit (Blood Sugar-GOD-Perid-Test, Boehringer, Mannheim, Federal Republic of Germany) essentially according to the method of Werner et al. (10). Mass and ^1H NMR spectra were measured with a Hitachi M-80 and a Nicolet NT-360, respectively.

RESULTS

Screening of fungal strains capable of producing EPA. Two hundred and fifteen strains of Mucoraceae were assayed as to their EPA productivity at a low growth temperature (12 C), because several arachidonic acid-producing *Mortierella* strains had been found to accumulate detectable amounts of EPA in their mycelia only when grown at low temperature (9). Such strains were found in the genus *Mortierella* at a high frequency. All f them (105 strains) produced arachidonic acid as another major C-20 polyunsaturated fatty acid. Through this test, we selected the 11 strains listed in Table 1, the EPA productivities at 12 C of which were compared with those at 28 or 24 C. In every case, EPA accumulation was observed only at 12 C, regardless f the medium used (i.e., medium GY or medium YM). The data in Table 1 show that these EPA producers fall roughly into two groups as to their cellular fatty acid profiles. One group includes most strains other than *M. alpina*. They accumulated oleic acid as the most predominant cellular fatty acid (20–60%). *M. alpina* strains are unique in that arachidonic acid is one of the most predominant cellular fatty acids (20–50%). In particular, arachidonic acid comprised nearly 50% f the total cellular fatty acids in *M. alpina* 1S-4 and 1-83 at 28 C. On the other hand, there was not such a clear difference as to EPA contents between these tw groups. We selected *M. hygrophila* and *M. alpina* 20-17 for the following experiments, because of their mycelial yields, lipid contents and EPA contents.

Effects of the growth temperature on the production of EPA. Both the strains grew well at l w temperature (6–16 C), although the growth rates were somewhat lower than those at higher temperature (20–28 C). The mycelial yields btained after five to seven days f cultivation in medium GY were comparable t

TABLE 2

Effect of the Growth Temperature on the Production of EPA[a]

Temperature (C)/ cultivation period (days)	Mycelial yield (mg/ml of culture medium)	Total FA[b] (mg/g dry mycelia)	Content of		Yield of		% in total FA[b]	
			EPA (mg/g dry mycelia)	Ara[b] (mg/g dry mycelia)	EPA (mg/ml of culture medium)	Ara[b] (mg/ml of culture medium)	EPA	Ara[b]
M. hygrophila IFO 5941								
6/11	7.7	148	15.3	11.1	0.12	0.09	10.3	7.5
12/6	9.7	163	14.6	16.3	0.13	0.16	6.5	8.1
16/6	8.7	220	6.9	26.8	0.06	0.23	3.1	12.2
20/6	7.7	167	6.2	31.6	0.04	0.24	3.7	18.9
28/6	7.4	133	0	75.6	0	0.17	0	17.6
M. alpina 20-17								
6/11	6.9	168	13.8	59.3	0.09	0.49	8.2	35.3
12/7	9.7	200	11.2	104.0	0.11	1.01	5.6	52.0
16/6	9.2	201	7.2	102.3	0.06	0.94	3.6	51.2
20/6	8.7	260	2.9	142.4	0.02	1.24	1.1	54.8
24/4	8.9	247	1.0	130.1	0.01	1.16	0.5	52.6
28/6	10.1	215	0	112.9	0	1.14	0	52.5

[a]Each strain was grown at various temperatures for 4 to 11 days, as indicated. Other conditions are given in the text.
[b]FA, fatty acid; Ara, arachidonic acid.

those on cultivation at higher temperature for four to six days. In both cases, the maximum EPA contents were obtained with mycelia grown at 6 C, as shown in Table 2. The maximum EPA production was, however, obtained at 12 C. Further elevation of the growth temperature brought about marked decreases in their EPA contents, although EPA accumulation was still observed up to 20 C. Conversely, their arachidonic acid contents increased with elevation of the growth temperature. The EPA content of *M. hygrophila* mycelia grown at 28 C was seven times higher than that at 6 C. It also should be noted that *M. alpina* is excellent in accumulating arachidonic acid. The arachidonic acid production reached 1.24 mg/ml of culture medium (142.4 mg/g dry mycelia) when cultivated at 20 C for six days. The arachidonic acid comprised more than 50% of the total mycelial fatty acid. A temperature shift from 28 to 12 C also induced EPA production. This occurred at any phase of growth, even when washed mycelia were allowed to stand at 12 C. When the temperature was shifted to 28 C after cultivation for four days at 12 C, no further increase in EPA accumulation was observed. More than 85% of the accumulated EPA during growth at 12 C remained unchanged on incubation of *M. hygrophila* for a further five days at 28 C. On the contrary, the EPA in *M. alpina* rapidly disappeared with the temperature shift (data not shown).

Other factors affecting EPA production. (i) Carbon sources. Various carbon compounds, including sugars, organic acids, alcohols, fatty acids, *n*-alkanes and oils, were tested as carbon sources in place of glucose in medium GY. Glycerol and maltose, and fructose were found to be effective carbon sources for *M. hygrophila* and *M. alpina*, respectively, producing almost the same mycelial yields and EPA contents as those with glucose. The EPA content of *M. hygrophila* grown with *n*-octadecane was double that with glucose, but the mycelial yield was poor (4.3 mg/ml). The optimum concentration of glucose at a fixed concentration of yeast extract (1%) was 2%, with which *M. hygrophila* and *M. alpina* accumulated 0.08 and 0.10 mg of EPA/ml of culture medium (11.1 and 11.3 mg/g dry mycelia), respectively. A further increase in the glucose concentration repressed the growth of both fungi.

TABLE 3

Effect of Glucose Feeding on the Production of EPA by *M. hygrophila* IFO 5941[a]

Cultivation period (days)	Day of feeding	Mycelial yield (mg/ml of culture medium)	Total FA[b] (mg/g dry mycelia)	EPA content (mg/g dry mycelia)	EPA yield (mg/ml of culture medium)
10		7.8	298	21.0	0.16
11	5,7,9	14.1	298	19.4	0.27
10	4,6,8	14.2	259	19.4	0.28
10	6,8	15.2	305	20.4	0.31

[a]*M. hygrophila* was grown in medium GY as indicated. Glucose was fed to maintain a level of 2% on the indicated days of cultivation. Compositions of medium GY and other conditions are given in the text.
[b]FA, fatty acid.

TABLE 4

Effect of a Temperature Shift and Glucose Feeding on the Production of EPA[a]

Cultivation period (days)	Glucose feeding	*M. hygrophila*				*M. alpina*			
		Mycelial yield (mg/ml of culture medium)	Total FA[b] (mg/g dry mycelia)	EPA content (mg/g dry mycelia)	EPA yield (mg/ml of culture medium)	Mycelial yield (mg/ml of culture medium)	Total FA[b] (mg/g dry mycelia)	EPA content (mg/g dry mycelia)	EPA yield (mg/ml of culture medium)
Exp. I (20 → 12 C)									
A 3	no	7.8	219	4.6	0.04	9.2	100	1.5	0.01
B 7	no	8.8	159	8.3	0.07	9.9	276	4.1	0.04
C 7	yes	8.0	388	17.8	0.14	6.7	96	29.1	0.20
D 7	yes	10.0	198	14.9	0.15	11.1	272	25.3	0.28
Exp. II (12 → 20 C)									
A 5	no	8.2	164	9.7	0.08	9.3	112	5.7	0.14
B 9	no	9.3	188	23.3	0.22	9.9	204	13.7	0.14
C 9	yes	7.1	610	40.9	0.29	7.1	308	25.0	0.18
D 9	yes	10.1	184	14.0	0.14	10.5	216	12.3	0.13

[a]In Experiment I, cultivation as performed in 4 flasks at the same time at 20 C. One of the flasks was analyzed on the 3rd day (A). The other three flasks were shifted to 12 C on the same day with (C and D) or without (B) glucose feeding to maintain the level of 2%; then, the cultivations were continued for a further 4 days. In D, yeast extract (0.2 mg/ml) also was added on the 3rd day. In Experiment II, the cultivations were started at 12 C. On the 5th day, one flask was analyzed (A). The other three flasks were shifted to 20 C with (C and D) or without (B) glucose feeding to maintain the level of 2%, and then the cultivations were continued for a further 4 days. In D, yeast extract (0.2 mg/ml) also was added on the 5th day. Other conditions are given in the text.
[b]FA, fatty acid.

(ii) Nitrogen sources. Various organic and inorganic compounds and natural nutrients were tested by adding each of them to medium GY to a final concentration of 0.5%, and by replacing the yeast extract in medium GY with 0.5% of each of them. Generally, the mycelial yields increased significantly on the addition of organic nitrogen sources such as tryptone, casamino acid, meat extract, etc., but the EPA contents of the two fungi decreased markedly. Only bactopeptone (Difco, Detroit, Michigan) was effective in increasing the mycelial yields without decreases in the EPA contents. In the replacement test, no nitrogen source superior to yeast extract was found, so far as tested. The optimum concentration of yeast extract at a fixed concentration of glucose (2%) was 1%.

(iii) Glucose feeding. Because the glucose in medium GY was consumed during the first four to five days of cultivation, and the presence of a high level of glucose at the initiation of the cultivation repressed the fungal growth, glucose was fed periodically to maintain a level of 2%. The data in Table 3 show that the mycelial mass increased about two-fold without a significant decrease in EPA content on feeding of glucose in every case tested. The maximum production of EPA (0.31 mg/ml, 20.4 mg/g dry mycelia) was attained on cultivation of *M. hygrophila* for 10 days with two successive feedings of glucose on the sixth and eighth days.

(iv) Combination of a temperature shift and glucose feeding. To reduce the growth period without any decrease in EPA producti n, each strain was grown under the c nditions (with glucose feeding and a temperature shift) shown in Table 4. The maximum EPA production with *M. alpina* (0.28 mg/ml) was obtained when the fungus was grown at 20 C for three days and then at 12 C for a further four days with feeding of glucose and yeast extract. This value was about seven times higher than that obtained under the conditions without feeding. More than 11 days were required to obtain essentially the same value when the fungus was grown without a temperature shift (data not shown). In the case of *M. hygrophila*, the maximum EPA production (0.29 mg/ml) was obtained when it was grown at 12 C for the first five days and then for a further four days at 20 C with glucose feeding. However, feeding of yeast extract led to decreased production of EPA.

EPA production under optimum culture conditions. Based on the above results, *M. alpina* was cultivated in medium GY supplemented with 0.5% bactopeptone for 11 days at 12 C with two successive feedings of glucose (1% each) on the seventh and nineth days. The EPA production reached 0.49 mg/ml of culture medium (29 mg/g dry mycelia). This value accounted for 13.5% of the total extractable mycelial fatty acids. Other major fatty acids were palmitic acid (6.0%), stearic acid (5.3), oleic acid (6.2), linoleic acid (3.0), γ-linolenic acid (3.5) and arachidonic acid (60.0). Essentially the same results were obtained when the cultivation was carried out at 20 C for the first three days followed by cultivation for a further six days at 12 C with two successive glucose feedings (1% each; fifth and seventh days).

Isolation of the EPA. The EPA methyl ester (6.7 mg) was isolated from the lipids extracted from 15 g of wet mycelia f *M. alpina* grown under optimum culture conditions. The mass spectrum f the is lated methyl ester showed a m lecular ion peak at m/z 316 (relative intensity, 7%) and intense fragment ion peaks at m/z 201, 180, 175, 173, 93, 91, 79 and 67 (relative intensity, 65, 100, 87, 52, 54, 74, 100 and 58%, respec-

tively). The ^1H NMR spectra in $CDCl_3$ with tetramethylsilane as an internal standard showed signals at 0.97 (*t*, 3H, CH_3), 1.72 (*m*, 2H, CH_2), 2.10 (*m*, 4H, CH_2), 2.33 (*t*, 2H, CH_2), 2.82 (*m*, 8H, CH_2), 3.67 (*s*, 3H, CH_3) and 5.38 ppm (*m*, 10H, C=C). These data corresponded well to those of authentic EPA methyl ester (Funakoshi Chemicals, Tokyo).

DISCUSSION

The results reported here show that fungal mycelia are rich sources of EPA. In particular, *M. alpina* 20-17 was found to accumulate about 0.5 mg of EPA/ml of culture medium (29 mg/g dry mycelia). There has been no report showing the possibility of fungal microorganisms being potential sources of it, before this work. These fungi are thought to be much more advantageous than the algal, moss and protozoal sources previously reported (3-6), because of their higher EPA contents, their higher growth rates in simple media and the simplicity of their manipulation. These features would make the use of these fungi as sources of EPA very promising.

All the EPA-producing strains found here accumulated polyunsaturated fatty acids of the n-6 series (i.e., γ-linolenic acid, dihomo-γ-linolenic acid and arachidonic acid) in their mycelia. However, no n-3 polyunsaturated fatty acid other than EPA was detected. This suggests that an n-6 PUFA may be a precursor of EPA. If this is the case, an enzyme(s) or enzyme system catalyzing the methyl-end directed desaturation (6,11) of one of these polyunsaturated fatty acids, probably arachidonic acid, may be formed or activated on cold adaptation. The resultant EPA may be necessary for maintaining a proper membrane fluidity in a low temperature environment. The mechanism underlying this cold-induced formation of EPA is now being studied (9).

For practical purposes, however, low temperature is disadvantageous because of low growth rate and high energy cost for cooling. The data in Table 4 and those obtained under optimal conditions suggest that combination of the temperature shift and glucose feeding during the growth of the fungi may be one of the effective ways of obtaining enough mycelia with high EPA content in a short cultivation period.

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Microbial Conversion of an Oil Containing α-Linolenic Acid to an Oil Containing Eicosapentaenoic Acid

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Mycelia of arachidonic acid-producing fungi belonging to the genus *Mortierella* were found to convert an oil containing α-linolenic acid to an oil containing 5,8,11,14,17-*cis*-eicosapentaenoic acid (EPA). This conversion was observed when they were grown in a medium containing the oil, glucose and yeast extract at 28 C. On the screening of various oils, linseed oil, in which α-linolenic acid amounts to about 60% of the total fatty acids, was found to be the most suitable for EPA production. Under the optimal culture conditions, a selected strain, *Mortierella alpina* 20-17, converted 5.1% of the α-linolenic acid in the added oil into EPA, the EPA production reaching 1.35 g/l of culture broth (41.5 mg/g dry mycelia). This value corresponded to 7.1% (by weight) of the total fatty acids in the extracted lipids. The lipid was also found to be rich in arachidonic acid (12.3%). Other major fatty acids in the lipid were palmitic acid (4.4%), stearic acid (3.2%), oleic acid (13.5%), linoleic acid (13.7%), α-linolenic acid (38.5%) and γ-linolenic acid (0.9%).

5,8,11,14,17-*cis*-Eicosapentaenoic acid (EPA) is a C-20 polyunsaturated fatty acid (PUFA) of potential pharmaceutical value. It has been shown to be effective in preventing or curing thrombosis (1–3).

EPA occurs as a component of cellular lipids in protozoal, algal, bacterial and animal cells (4–7). Several marine fish oils have become available recently as sources of EPA, but are not satisfactory for practical purposes because of their low EPA contents, variability of the EPA content between catches and the presence of other fatty acids with less desirable properties.

In recent studies, we have found that arachidonic acid-producing fungi belonging to the genus *Mortierella* accumulate EPA in their mycelia when grown in conventional media containing glucose as the major carbon source at low temperature (6–20 C), and that the resultant mycelia are rich in EPA and arachidonic acid (8–13). We have also suggested that this production may be due to activation of the enzyme(s) involved in EPA formation, probably in the methyl-end directed desaturation of arachidonic acid to EPA, at low temperature (11) (Fig. 1).

In a mammalian system, on the other hand, it has been demonstrated that dietary α-linolenic acid is converted to EPA through the n-3 route, as shown in Figure 1 (14). If the same route occurs in the *Mortierella* fungi, it may be a very promising route for the practical production of EPA, because there are various kinds of natural oils containing α-linolenic acid that are easily available, and these oils may be expected to be converted to EPA-containing oils on incubation with these fungi. The present study was carried out to examine the potential of such natural oils as precursors of EPA. The data presented here show that several arachidonic acid-producing *Mortierella* fungi effectively convert α-linolenic acid-containing oils, such as linseed oil, with the accumulation of EPA-containing oils in the mycelia.

MATERIALS AND METHODS

Chemicals. Linseed oil was purchased from Wako Pure Chemicals, Osaka, Japan. The oil contained palmitic acid (10.0%, by weight), stearic acid (3.7%), oleic acid (10.7%), linoleic acid (17.5%) and α-linolenic acid (58.1%). Fungal oil was prepared from mycelia of *Mortierella alpina* 1S-4 as described (11). Perilla oil, fish oil and the other oils listed in Table 2 were obtained from Yamakei Sangyo, Osaka, Japan, Toyo Jozo Co., Tokyo, Japan, and Sigma Chemical Co., St. Louis, Missouri. The fatty acid compositions, by weight, of these oils are given in the footnote to Table 2. Methyl α-linolenate was purchased from Funakoshi Chemicals, Tokyo, Japan. All other chemicals used in this work were as described previously (12).

Microorganisms, media and cultivations. All fungal strains used were from our stock cultures (AKU Culture Collection, Faculty of Agriculture, Kyoto University). Each fungus was inoculated into a 50-ml shaking flask containing 10 ml of medium GY (13) containing 1% oil, followed by incubation at 28 C for six days with reciprocal shaking (120 strokes/min), unless otherwise stated.

Fatty acid analysis and other methods. Fungal mycelia were harvested by suction filtration, washed with 50 ml of ether acidified with 0.5 ml of 2 N HCl and then with 50 ml of water. The filtered mycelia were dried at

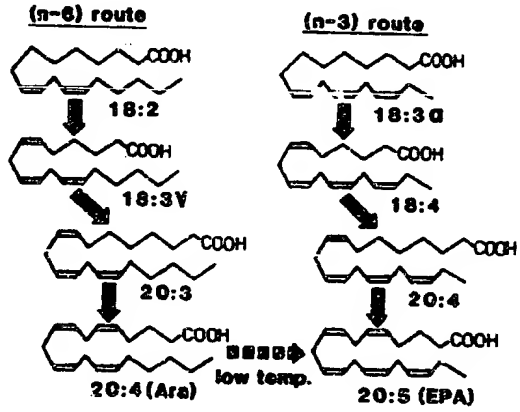

FIG. 1. Pathways for the biosynthesis of arachidonic acid and EPA. 18:2, linoleic acid; 18:3α, α-linolenic acid; 18:3γ, γ-linolenic acid; 18:4, 6,9,12,15-octadecatetraenoic acid; 20:3, dihomo-γ-linolenic acid; 20:4, 8,11,14,17-eicosatetraenoic acid; 20:4 (Ara), arachidonic acid; 20:5 (EPA), eicosapentaenoic acid.

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TABLE 1

EPA Production by *M. alpina* 20-17 on Incubation on a Glucose-Yeast Extract Medium with α-Linolenate and/or Olive Oil at 28 C[a]

Supplement	Mycelial yield (mg)[b]	Total FA[c] (mg)[b]	EPA content (mg/g dry mycelia)	EPA yield (μg)[d]
Exp. I				
None	3.5	0.19	0.	0.
Methyl-α-linolenate (0.5%)	5.1	0.53	1.8	8.9
Olive oil (1.5%)	7.0	2.28	0.	0.
Olive oil (1.5%) + methyl-α-linolenate (0.2%)	10.3	3.20	1.2	12.4
Olive oil (1.5%) + methyl-α-linolenate (0.5%)	10.5	3.47	3.4	35.3
Olive oil (1.5%) + methyl-α-linolenate (1.0%)	15.1	4.75	3.5	52.3
Exp. II				
None	10.4	1.52	0.	0.
Olive oil (1.5%)	16.4	4.31	0.	0.
Olive oil (1.5%) + methyl-α-linolenate (0.2%)	19.0	5.09	3.3	63.1

[a]In Exp. I, *M. alpina* 20-17 was grown on 2% agar plates containing medium GY (2% glucose and 1% yeast extract, pH 6.0) and the supplements indicated for 15 days. In Exp. II, the organism was grown in medium GY with the supplements indicated for 9 days with shaking.
[b]Values are given in mg obtained from 1 g (Exp. I) or 1 ml (Exp. II) of culture broth.
[c]FA, fatty acid.
[d]Values are given in μg found in 1 g (Exp. I) or 1 ml (Exp. II) of culture broth.

TABLE 2

EPA Production by *M. alpina* 20-17 in the Presence of Several Natural Oils[a]

Oil added[b]	Productivity: Mycelial mass (mg/ml of culture broth)	Productivity: EPA (mg/ml of culture broth)	Productivity: Ara[d] (mg/ml of culture broth)	Fatty acid composition (%)[c]: 16:0	18:0	18:1	18:2	18:3α	18:3γ	20:3	20:4	20:5	others
Linseed	15.9	0.25	1.63	6.1	3.3	9.2	9.4	27.8	2.1	3.0	33.8	5.1	0.2
Soybean	19.7	0.02	1.32	8.4	3.8	21.0	40.8	5.3	2.8	2.4	14.8	0.3	0.4
Perilla	15.5	0.18	1.32	8.9	3.6	14.2	11.0	26.6	2.0	2.1	27.6	3.8	2.2
Olive	17.0	0.	1.36	9.5	3.1	49.3	14.2	0.	3.0	3.0	16.2	0.	1.7
Evening primrose	15.3	0.	1.36	9.3	2.4	10.1	45.6	0.	4.9	5.0	21.4	0.	1.3
Peanut	18.1	0.	1.00	8.1	5.1	35.1	27.7	0.	3.8	5.2	12.4	0.	2.6
Mortierella	19.2	0.	2.14	12.8	6.1	37.7	10.6	0.	3.3	5.2	22.7	0.	1.6
Fish	14.4	0.26	0.91	16.0	3.2	20.6	8.1	tr[e]	5.4	tr	27.3	7.9	11.5
None	10.5	0.	0.63	15.9	8.1	21.0	14.3	0.	5.5	4.7	28.4	0.	2.1

[a]*M. alpina* 20-17 was grown in medium GY under the conditions in Materials and Methods with the addition of each oil (1.0%), as indicated.
[b]The fatty acid compositions of the oils, in wt %, were as follows: soybean oil, 16:0, 10.0; 18:0, 3.7; 18:1, 23.1; 18:2, 53.8; 18:3, 8.8; others, 0.6; perilla oil, 16:0, 7.1; 18:0, 2.0; 18:1, 14.1; 18:2, 14.5; 18:3α, 62.3; olive oil, 16:0, 9.9; 18:0 + 18:1, 82.2; 18:2, 6.4; 18:3α, 1.6; evening primrose oil, 16:0, 6.7; 18:0, 1.9, 18:1, 10.2; 18:2, 68.9; 18:3γ, 11.3; others, 1.0; peanut oil, 16:0, 14.4; 18:0, 2.5; 18:1, 50.8; 18:2, 32.3; *Mortierella* oil, 16:0, 19.4; 18:0, 5.3, 18:1, 42.8; 18:2, 9.3; 18:3γ, 2.5; 20:3, 2.6; 20:4, 15.4; others, 2.7; fish oil, 16:0, 15.6; 16:1, 8.5; 18:0, 2.5; 18:1, 16.8; 18:2, 1.9; 20:5, 16.1; 22:6, 20.0; others, 20.4. For linseed oil, see the text.
[c]16:0, palmitic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3α, α-linolenic acid; 18:3γ, γ-linolenic acid; 20:3, dihomo-γ-linolenic acid; 20:4, arachidonic acid; 20:5, EPA.
[d]Ara, arachidonic acid.
[e]tr, trace.

100 C overnight. Transmethylation of the sample in methylene chloride-10% methanolic HCl and then extraction of the fatty acid methyl esters with *n*-hexane were carried out with *n*-heptadecanoic acid as an internal standard as described previously (12,13). The resultant esters were analyzed by gas liquid chromatography (GLC) (12,13). The analysis well resolved all PUFAs of the n-3 and n-6 families. Mycelial fatty acid composition values are given in weight %. Each PUFA f the n-3 family found in mycelia was usually less than 1% of total fatty acids except for α-linolenic acid and EPA. Compositions of these minor PUFAs are summed up and given as "others" in each table.

Productivity of EPA usually was represented by using two indexes, mg of EPA/g of dry mycelia and mg f EPA/ml of culture broth, which, respectively, indicate mycelial content of EPA and total EPA produced in one ml of culture broth.

The methods used for fungal growth measurement and mass and ^{1}H NMR spectral analyses were described

TABLE 3

Comparison of EPA Productivities and Mycelial Fatty Acid Compositions in *Mortierella* Fungi Grown with Linseed Oil[a]

	Productivity			Fatty acid composition (%)[b]									
	Mycelial mass (mg/ml of culture broth)	EPA	Ara[b]	16:0	18:0	18:1	18:2	18:3α	18:3γ	20:3	20:4	20:5	others
M. alpina 20-17 AKU 3996	15.1	0.24	1.12	10.6	4.9	11.2	13.0	22.3	2.8	3.0	22.9	4.9	4.4
M. alpina 1S-4 AKU 3998	29.2	0.11	0.82	9.0	3.8	19.7	16.9	42.0	tr[c]	1.2	6.1	0.8	0.5
M. alpina 1-83 AKU 3995	26.8	0.14	0.71	12.4	4.6	23.9	14.5	30.9	1.2	1.6	8.2	1.7	1.0
M. alpina CBS 250.53	10.3	0.01	0.21	10.5	5.1	23.2	15.2	35.0	2.1	1.5	6.8	0.3	0.3
M. elongata 1S-5 AKU 3999	14.5	0.05	0.33	10.4	6.9	21.7	12.5	37.2	2.2	1.9	5.6	0.9	0.7
M. elongata IFO 8570	13.2	0.05	0.34	8.4	6.5	20.5	13.5	38.5	2.0	1.5	7.5	1.2	0.4
M. elongata NRRL 5513	14.9	0.33	0.28	11.1	6.4	23.3	13.6	35.3	2.2	1.3	5.5	0.6	0.7
M. parvispora AKU 3994	11.0	0.02	0.21	7.2	5.1	23.7	14.0	40.6	1.8	1.2	5.3	0.4	0.7
M. beljakovae CBS 601.68	14.7	0.01	0.11	7.7	3.8	19.0	17.2	48.4	tr	0.7	2.3	0.2	0.7
M. epigama CBS 489.70	13.5	0.01	0.10	11.0	5.6	23.0	14.8	38.2	1.7	0.8	3.8	0.3	0.8
M. verticillata IFO 8575	12.9	0.02	0.22	12.4	5.7	25.1	13.5	31.6	2.6	1.7	5.7	0.6	1.1
M. hygrophila IFO 5941	8.7	0.02	0.15	13.0	6.1	25.6	12.1	30.1	3.3	2.0	6.2	0.8	0.8
M. kuhlmanii CBS 157.71	15.5	0.03	0.28	11.4	7.5	20.9	13.3	37.8	1.3	1.3	4.8	0.5	1.2
M. zychae CBS316.52	14.9	0.03	0.22	9.1	5.1	19.1	16.0	45.5	0.7	0.8	3.0	0.4	0.3
M. bainieri IFO 8569	8.9	0.03	0.18	14.9	6.9	24.6	11.5	27.2	2.7	2.8	7.8	1.2	0.4
M. minutissima IFO 8573	13.0	0.01	0.15	12.0	6.4	22.8	13.8	30.9	2.9	1.7	4.7	0.4	4.4
M. bisporalis NRRL 2493	5.5	0.02	0.05	7.3	2.8	16.7	18.4	47.3	1.6	0.5	3.0	1.0	1.4
M. schmuckeri NRRL 2761	14.4	0.01	0.23	13.3	8.1	27.3	12.7	29.5	1.9	1.9	4.4	0.2	0.7
M. hyalina NRRL 6427	11.5	0.03	0.19	11.5	7.0	23.1	13.4	35.5	2.3	1.4	4.5	0.7	0.6
M. sp. NRRL 1458	9.8	0.01	0.12	14.3	6.5	26.1	13.1	28.6	5.6	1.5	5.8	0.5	0.2

[a]Each strain was grown under the conditions in Materials and Methods.
[b]Abbreviations for fatty acids are given in the footnotes to Table 2.
[c]tr, trace.

previously (12). Glucose concentrations were determined with a commercially available kit (Blood Sugar-GOD-Perid-Test, Boehringer, Mannheim, Germany), essentially according to the method of Werner et al. (15).

RESULTS

EPA production by M. alpina *20-17 during growth on medium supplemented with α-linolenic acid.* As described previously, most *Mortierella* strains capable of producing arachidonic acid produce EPA when grown at low temperature (6-16 C), but not at 28 C (8-12). Because these fungi cannot produce α-linolenic acid (11-13), we examined whether or not added α-linolenate is converted to EPA at this temperature. Under all the tested conditions when α-linolenate was present, detectable amounts of EPA were found in the mycelia of *M. alpina* 20-17, suggesting that the addition of α-linolenate is indispensable for EPA production at 28 C (Table 1).

Screening of suitable oils as starting substrates for EPA production. Because α-linolenic acid itself is a rather expensive reagent as a practical precursor of EPA, we examined several easily available natural oils as sources of α-linolenic acid. The results in Table 2 show that *M. alpina* 20-17 accumulated a detectable amount of EPA in its mycelia on incubation with linseed oil, perilla oil or soybean oil, all of which contain α-linolenic acid as a major fatty acid. EPA was also found in the mycelia grown with fish oil which contains EPA as a major fatty acid. However, the amount found after the cultivation was only about 50% of that initially added to the medium. EPA was not detected in any mycelia obtained with the other oils tested, which contain no or only very small amounts of α-linolenic acid (less than 3% of the total fatty acids). On the other hand, high amounts of arachidonic acid were found after the cultivation, regardless of the type of oil. These results again indicate that the presence of α-linolenic acid can result in EPA production. The α-linolenic acid probably was converted to EPA during the growth of the fungi. The highest accumulation of EPA was found in the mycelia obtained with linseed oil. The EPA content reached 15.7 mg/g dry mycelia (0.25 mg/ml). This value corresponded to a molar conversion ratio of added α-linolenic acid to EPA of about 4%.

Selection of Mortierella *strains showing high EPA productivity.* Arachidonic acid-producing *Mortierella* fungi (20 strains) obtained in the previous studies were compared as to their EPA productivity in medium GY supplemented with 1% linseed oil at 28 C. All were confirmed not to produce EPA at 28 C in the unsupplemented medium, although they produce it at a low growth temperature (6-16 C) (11,12,16). The data in Table 3 show that all the tested strains produced detectable amounts of EPA on incubation with linseed oil at 28 C. In particular, several *Mortierella alpina* strains were found to accumulate EPA at more than 3.0 mg/g dry mycelia. These *M. alpina* strains were also excellent at production of arachidonic acid in the medium either with or without linseed oil [for the data without linseed oil, see (12) and (13)]. On the other hand, some *Mortierella* strains (21 strains) only produced fatty acids with 18 or less carbon atoms (i.e., palmitic acid, stearic acid, oleic acid, linoleic acid and γ-linolenic acid), that is, not C-20 PUFAs such as dihomo-γ-linolenic acid or arachidonic acid, on incubation with linseed oil (data not shown). Through this test, we selected three strains of *M. alpina* (i.e., 20-17, 1S-4 and 1-83) for the following studies, on the basis of their high EPA productivities at 28 C.

Factors affecting EPA production. (i) Time course

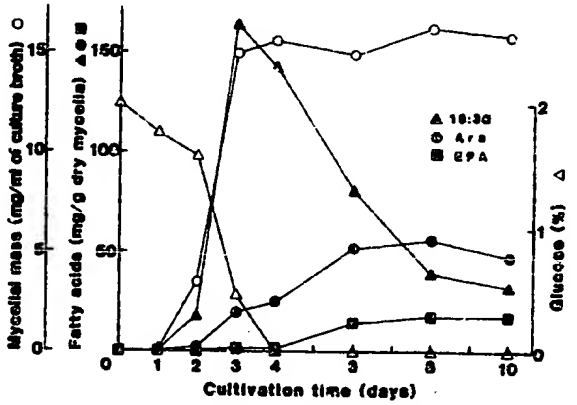

FIG. 2. Effect of the culture time on the production of EPA. *M. alpina* 20-17 was grown under the conditions in Materials and Methods except for the cultivation time as indicated. 18:3α, α-linolenic acid; Ara, arachidonic acid.

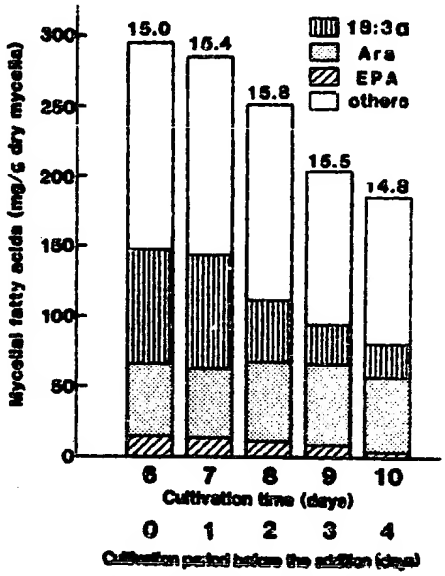

FIG. 3. Effect of the time of linseed oil addition on the production of EPA. *M. alpina* 20-17 was grown under the conditions in Meterials and Methods except for the time of addition of the oil (1%) and the cultivation time, as indicated. The values given on the top of each bar indicate mycelial mass (mg/ml) after cultivation. 18:3α, α-linolenic acid; Ara, arachidonic acid.

of EPA formation. First, the changes in the concentrations of glucose and α-linolenic acid in the linseed oil in the growth medium, and those in the mycelial contents of α-linolenic acid, arachidonic acid and EPA during the growth of *M. alpina* 20-17 in medium GY supplemented with 1% linseed oil were monitored (Fig. 2). The α-linolenic acid and other fatty acids in the linseed oil in the culture filtrate were undetectable when analyzed on the third day of cultivation, suggesting that they were almost completely consumed by the fungus during the first three days. During the same period, 75% of the added glucose was consumed, the mycelial yield being 15.0 mg/ml of culture broth. Eighty percent of the α-linolenic acid was found in the mycelia at this point. But the EPA content of the mycelia was only 1.6 mg/g dry mycelia. This value corresponds to only 0.5% of the total extracted fatty acids. Other

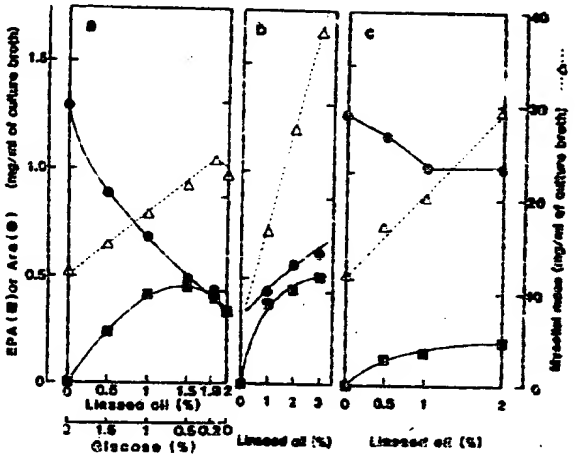

FIG. 4. Effect of the linseed oil concentration on the production of EPA. *M. alpina* 20-17 was grown for 6 days at 28 C. The concentrations of glucose and linseed oil in the medium were varied as shown. In b and c, initial glucose concentrations were 0.5% and 2.0%, respectively. In all cases, the medium contained 1% yeast extract. Other conditions are given in Materials and Methods. Ara, arachidonic acid.

major fatty acids found in the mycelia were palmitic acid (9.9%), stearic acid (5.7%), oleic acid (18.1%), linoleic acid (15.1%), α-linolenic acid (42.9%) and arachidonic acid (5.2%). After eight days, mycelia containing 17.9 mg EPA/g dry mycelia were obtained, the mycelial yield being 16.1 mg/ml of culture broth. The predominant fatty acids in the extracted lipids were as follows: palmitic acid (10.9%), stearic acid (5.7%), oleic acid (12.8%), linoleic acid (11.5%), α-linolenic acid (17.5%), arachidonic acid (25.1%) and EPA (7.5%).

(ii) Time of the addition of linseed oil. The mycelial EPA content varied markedly, depending on the growth phase at which the oil was added. The data in Figure 3 indicate that addition at an early phase (i.e., 0 to 3 days after inoculation) is effective for high EPA production. The level of mycelial α-linolenic acid was only 23.4 mg/g dry mycelia, and about 30% of the added α-linolenic acid remained in the culture filtrate when the oil was added on the fourth day of cultivation and analysis was performed after the mycelia had been cultivated for a further six days. These results suggest that the low EPA productivity on delayed addition of linseed oil is due to the low efficiency of the mycelia to incorporate the added oil.

Relationship between the linseed oil and glucose concentrations. The EPA production at various concentrations of linseed oil was compared, as shown in Figure 4. The presence of glucose in the medium is not essential for EPA production, because *M. alpina* 20-17 used the added linseed oil as either a carbon source for growth or a precursor of EPA, and produced enough mycelia rich in EPA (Fig. 4a). The amount of EPA/ml of culture broth increased with increasing linseed oil concentration (Fig. 4b and c). Under the conditions of 3% linseed oil, 0.5% glucose and 1% yeast extract, the amount of EPA accumulated reached 0.52 mg/ml of culture broth. It should be noted that a high concentration of glucose (2.0%) repressed the EPA production; the amount of EPA accumulated with 2% linseed oil

TABLE 4

Changes in Mycelial EPA Content during Aging of Harvested Mycelia[a]

Strain	Mycelial mass (mg/ml of culture broth)	PUFA content: EPA (mg/g dry mycelia)	PUFA content: Ara[b] (mg/g dry mycelia)	Fatty acid composition (%)[b]: 16:0	18:0	18:1	18:2	18:3α	18:3γ	20:3	20:4	20:5	others
M. alpina 20-17													
Condition I													
Before aging	32.8	26.5	49.6	4.7	3.8	13.9	14.8	45.5	1.0	1.1	8.0	4.3	2.9
After aging	32.4[c]	41.5	71.5	4.4	3.2	13.5	13.7	38.5	0.9	1.4	12.3	7.1	5.0
Condition II													
Before aging	55.0	25.3	50.8	4.5	3.8	14.2	15.1	45.0	1.3	1.2	7.5	3.7	3.7
After aging	32.1	30.5	60.9	7.1	2.4	13.8	14.7	37.1	1.6	1.5	11.0	5.5	5.3
M. alpina 1S-4													
Condition I													
Before aging	21.8	15.8	33.3	7.7	3.6	14.9	14.3	44.7	1.3	1.3	6.7	3.2	2.3
After aging	23.1[c]	36.3	56.8	6.8	3.1	13.7	13.0	35.0	1.5	1.6	13.3	8.5	3.5
Condition II													
Before aging	22.5	16.3	30.0	7.6	3.3	14.9	14.8	45.4	1.0	1.0	7.0	3.8	1.4
After aging	23.4	20.1	39.3	6.8	2.3	13.4	13.8	37.2	1.3	1.4	12.9	6.6	4.3
M. alpina 1-33													
Condition I													
Before aging	21.2	23.4	57.5	7.1	5.0	13.4	12.5	35.9	1.4	2.6	14.0	5.4	2.7
After aging	22.2[c]	39.9	78.4	5.0	3.6	12.9	12.3	31.6	1.3	2.1	18.5	9.4	3.3
Condition II													
Before aging	22.8	20.4	61.0	7.5	3.5	15.0	15.0	41.7	1.2	1.4	10.0	3.3	1.4
After aging	22.3	26.3	70.1	7.0	2.7	14.7	14.4	37.5	1.0	1.4	11.9	4.1	5.3

[a]Each strain was cultivated for 6 days under the conditions in Materials and Methods except for the glucose concentration (4%) in the case of *M. alpina* 20-17. In Condition I the mycelia, after removal of the medium by suction filtration, were divided into two portions, one of which was analyzed for fatty acids and the other allowed to stand for a further 7 days at 28 C to age before being analyzed. In Condition II, the mycelia after removal of the medium were divided into two portions of equal weight, one of which was analyzed for fatty acids and the other incubated in 5 ml of culture filtrate for a further 7 days at 28 C. All operations were carried out under sterile conditions.

[b]Abbreviations for fatty acids are given in the footnotes to Table 2.

[c]Mycelial weight corresponding to that obtained from 1 ml of culture broth.

was only 0.21 mg/ml of culture medium. This value is about one-half of that attained under the conditions of a low glucose concentration (0.5%) with the same linseed oil concentration. On the other hand, arachidonic acid production was markedly enhanced with a high glucose concentration; for example, the amount of arachidonic acid (1.03 mg/ml) obtained under the conditions of 2% glucose and 2% linseed oil was about two-fold higher than that obtained under the conditions of 0.5% glucose and 2% linseed oil (Fig. 4b and c).

(iv) Growth temperature. The EPA production and mycelial yield in medium GY containing 1% linseed oil with various growth temperatures were investigated. The maximum EPA production (0.22 mg/ml) by *M. alpina* 20-17 was attained at 28 C on six days cultivation. This was suggested to be due mainly to the rapid growth of the fungus at this temperature (mycelial mass, 16.2 mg/ml), because the mycelial EPA content did not vary significantly at any temperature tested. To obtain the same mycelial yield, a further three or two days was required at 20 or 30 C, respectively (data not shown). At 35 C, the fungus could not grow under the conditions tested.

Increase in mycelial EPA content during aging of the harvested mycelia. The data in Table 4 show that EPA in the harvested mycelia can be specifically enriched when the mycelia are allowed to stand for a further few days. This phenomenon was observed for every *M. alpina* strain tested. The EPA content of the mycelia of *M. alpina* 20-17 which were collected on the sixth day of cultivation and then allowed to stand for a further seven days at 28 C reached 41.5 mg/g dry mycelia; this value was about 1.6 times higher than that determined at the initiation of this aging. Because no significant change in mycelial weight was observed on the aging, the corresponding value, in mg/ml of culture broth, was calculated to be 1.35, suggesting that 0.48 mg of EPA was newly produced by the mycelia obtained from one ml of culture medium. A similar increase was observed in arachidonic acid. As a result, EPA and arachidonic acid comprised 7.1 and 12.3% of the total mycelial fatty acids, respectively. On continuing cultivation for the same period in the liquid medium without shaking, the increase in the EPA content was only 20-30% of the content before the aging (Table 4).

The EPA methyl ester (16 mg) was isolated from the lipids extracted from 20 g of wet mycelia of *M. alpina* 20-17, which was grown for six days under the conditions given in Table 4 and aged for a further seven days, according to essentially the same procedure as described previously (13). The mass and ^1H NMR spectra of the isolated methyl ester corresponded well to those previously reported (12).

DISCUSSION

W have reported that most arachidonic acid-produc-

ing *Mortierella* fungi can produce EPA when grown at low temperatur (6-16 C) (8-12). The n-6 route to arachidonic acid from a common C-18 fatty acid, linoleic acid, followed by the methyl-end directed desaturation of the arachidonic acid has been suggested for the route to EPA (11). The present finding that mycelia of the same fungi can produce EPA on incubation with α-linolenic acid, added as the methyl ester or an oil, at high temperature (20-30 C) suggested that there is also a temperature-independent route to EPA through which the added α-linolenic acid is converted to EPA, in these fungi. The most probable route for this EPA production is the n-3 route, which involves the following three successive reactions; Δ6-desaturation of α-linolenic acid to octadecatetraenoic acid (18:4 n-3), elongation to eicosatetraenoic acid (20:4 n-3) and Δ5-desaturation of the eicosatetraenoic acid to EPA, as shown in Figure 1. It has been reported that both the n-6 and n-3 routes share enzymes concerned in Δ6-desaturation, elongation and Δ5-desaturation in a mammalian system (17). If the same mechanism occurs in the *Mortierella* fungi reported here, the added α-linolenic acid, in place of linoleic acid, could be desaturated and converted to EPA through the n-3 route. The findings reported here that only the *Mortierella* strains producing arachidonic acid can produce EPA on incubation with linseed oil containing α-linolenic acid and that the strains which cannot produce C-20 PUFAs of the n-6 route, i.e., dihomo-γ-linolenic acid and arachidonic acid, cannot produce EPA under the same conditions may be consistent with the above assumption. The results shown in Figure 4, i.e., the elevation of arachidonic acid production with repressed production of EPA on incubation with a high concentration of glucose and the increased production of EPA with repressed production of arachidonic acid on incubation with a high concentration of α-linolenic acid, may be explained by this sharing of enzymes by the two routes. Under the conditions used here, linoleic acid is thought to be produced mainly from glucose, while α-linolenic acid is derived soley from the added linseed oil. Therefore, a high concentration of glucose is probably effective in increasing only the linoleic acid content in the mycelia. As a result, these two fatty acids would compete with each other as the substrate for the enzyme catalyzing Δ6-desaturation.

The ability of the *Mortierella* fungi to convert added α-linolenic acid to EPA is very promising from a biotechnological viewpoint because there are various kinds of easily available natural oils containing α-linolenic acid, and it is expected that they can be converted to oils rich in EPA on incubation with these fungi. Indeed, the incubation of linseed oil with *M. alpina* 20-17 resulted in the production of a novel oil rich in EPA and arachidonic acid. The EPA production obtained here (1.35 mg/ml) is 2.8-fold higher than that obtained under low temperature growth conditions. Another advantage of the present EPA production is that it can be carried out under normal growth temperature conditions (20-30 C). Under such conditions, the fungal growth is rapid and dense, and the energy costs for temperature control may be less than those for cooling.

Increase in mycelial contents of EPA and arachidonic acid by aging of the harvested mycelia as shown in Table 4 indicates that these PUFAs were still synthesized actively after cultivation in the liquid medium had stopped. Because an increase in PUFAs on continuing cultivation in the liquid medium with or without shaking was not so prominent, some environmental change which causes enhancement of PUFA production might be induced by this treatment. For practical purposes, this aging is very useful to enrich EPA content of harvested mycelia.

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Bajpai et al., "Effects of Aging Mortierella Mycelium on Production of Arachidonic and Eicosapentaenoic Acids", pp. 775-780, 1991, JAOCS, vol. 68, Oct.

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Gandhi et al., pp. 1825-1830, 1991, J. Gen. Microbiol., vol. 137

The Filamentous Fungus *Mortierella alpina*, High in Arachidonic Acid

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Arachidonic acid is not available readily, although it is widely distributed in animal tissue. We found that in some strains of *Mortierella alpina*, arachidonic acid accounted for 68.5–78.8% of the total fatty acids. This is more than twice the arachidonic acid content of any organism previously reported. The content of arachidonic acid per dry cell weight was about 25%. Our findings offer a method for the efficient isolation of arachidonic acid in large amounts.
***Lipids* 22, 1060–1062 (1987).**

Arachidonic acid is present widely in the animal kingdom. It has been isolated from the lipids extracted from pig adrenal gland or pig liver and from sardines, which also are an important source of eicosapentaenoic and docosahexaenoic acids. However, the arachidonic acid content is usually less than 5%, and the yield per dry weight is 0.2% or lower. Hence, large scale production of the acid is difficult.

Methods for culturing microorganisms that would be capable of producing larger amounts of arachidonic acid (1) have been proposed. Iizuka (2) reported on arachidonic acid-producing microorganisms belonging to the genera *Penicillium, Cladosporium, Mucor, Fusarium, Hormodendram, Aspergillus* and *Rhodotorura* that are cultured in media containing hydrocarbons or carbohydrates as carbon sources. This approach can produce an arachidonic acid content up to 7.5% of total lipid weight or less than 1% of dry cell weight. It also has been reported (3) that some strains of the genera *Entomophthora, Delacroixia, Conidiobolus, Pythium* and *Phytophthera* produce relatively high arachidonic acid levels. Arachidonic acid amounted to 27.1% of total fatty acid in *Entomophthora exitialis*, 19.1% in *E. ignobilis* and 18.8% in *E. thaxteriana*. It also has been shown (4) that dried mycelia of *Mortierella renispora* contain 4.8% of lipids, 26.7% of which is arachidonic acid. Ahern (5,6) showed that the red alga *Porphyridium cruentum* produces arachidonic acid with a potential yield of 1–8% of the total dry cell weight.

However, the arachidonic acid content in these species still is not sufficiently high. Hence, it was this study's aim to find microorganisms that would be capable of more efficiently producing arachidonic acid-containing lipids.

MATERIALS

The Institute for Fermentation Osaka, Japan (IFO), and American Type Culture Collection (ATCC) Rockville, MD, supplied the strains of *Mortierella alpina* used: IFO 8568, ATCC 16266, ATCC 32221, ATCC 42430.

Methyl arachidonate (purity 99%) was purchased from Nu Chek-Prep. Inc., Elysian, MN. Malt agar medium was purchased from Nissui Pharmaceutical Co., Tokyo, Japan. Reversed-phase thin layer chromatographic (TLC) plates, RP-18F, were a product of Merck, Darmstadt, West Germany.

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Abbreviations: DEGS, diethyleneglycol succinate; TLC, thin layer chromatography.

Gas-liquid chromatography was carried out using a Hitachi 663-50 gas chromatograph and a 1.5-m glass column of 3 mm bore packed with diethyleneglycol succinate (DEGS) 15%, SE-30 or OV-101. The instrument was fitted with a flame ionization detector. Gas chromatography-mass spectrometry was done on a Hitachi M-80 and followed by data analysis on a Hitachi M-003. ^{1}H-NMR and ^{13}C-NMR spectra were measured using a JEOL Model FX-90Q instrument.

METHODS

Ninety g of malt agar medium was added to 2,000 ml of distilled water and autoclaved for 15 min. The resulting medium was poured into 100 sterilized dishes that were 80 mm in diameter. Four strains of *M. alpina* (IFO 8568, ATCC 16266, ATCC 32221 and ATCC 42430) were inoculated each on 25 malt agar plates and incubated at 25 C for 20 days. On the 6th, 9th, 12th, 15th and 20th day after inoculation, mycelia of IFO 8568 growing on five plates were collected with a spatula and measured for growth and lipid changes. The mycelia of the other cultures similarly were collected, dried in an evacuated desiccator and then crushed by mortar and pestle using chloroform/methanol (2:1, v/v). The lipids were extracted with chloroform/methanol (2:1, v/v), and the solvent was evaporated. The lipids obtained then were converted to methyl esters with sodium methoxide. The fatty acid composition of the esters was analyzed by gas chromatography.

For the preparative purification of arachidonate, 50 mg of methyl esters that was obtained by the esterification of the total lipids from *M. alpina* IFO 8568 was subjected to reversed-phase TLC RP-18F using methanol/acetonitrile (1:1, v/v) as a developing solvent. A band at Rf 0.41 was scraped off, and the lipids were recovered to give 35 mg of methyl arachidonate with a purity of 95.9% (the remaining 4.1% being methyl γ-linolenate).

RESULTS AND DISCUSSION

Mycelium was not observed in any of the cultures until the third day after inoculation. Mycelia collected from fiv plates of *M. alpina* IFO 8568 from the 6th, 9th, 12th, 15th and 20th day of incubation gave the growth results, methyl esters profiles, and the increase in arachidonic acid shown in Figure 1.

The fungus growth reached a maximum at about 12 days, as did the amounts of total methyl esters and arachidonate. However, the percentage of arachidonate in the methyl esters reached a plateau three days later. When fatty acids other than arachidonic acid were produced beyond a certain level, it appears that excess acids were metabolized to arachidonic acid.

Figure 2 shows the changes in the composition of fatty acid methyl esters btained from IFO 8568 mycelia between six and 20 days. The sum of the six methyl ester percentages at each incubation time stayed near 92.4 ± 1.5%. Arachidonic acid increased dramatically throughout the incubation whereas palmitic, stearic, oleic, linoleic and γ-linolenic acids decreased. The content f α-linolenic

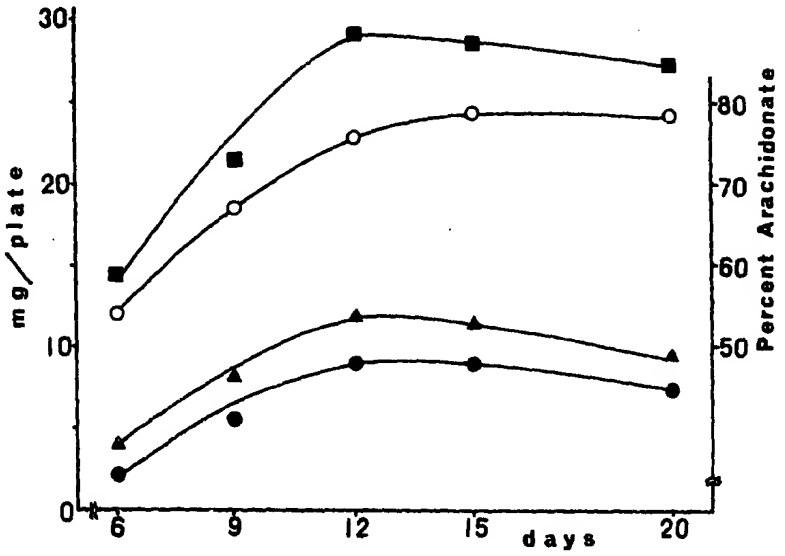

FIG. 1. Growth of *Mortierella alpina* IFO 8568 on malt agar plates at 25 C. Dry cell weight (■), weight of total methyl esters (▲), weight of methyl arachidonate (●) and content of methyl arachidonate (○).

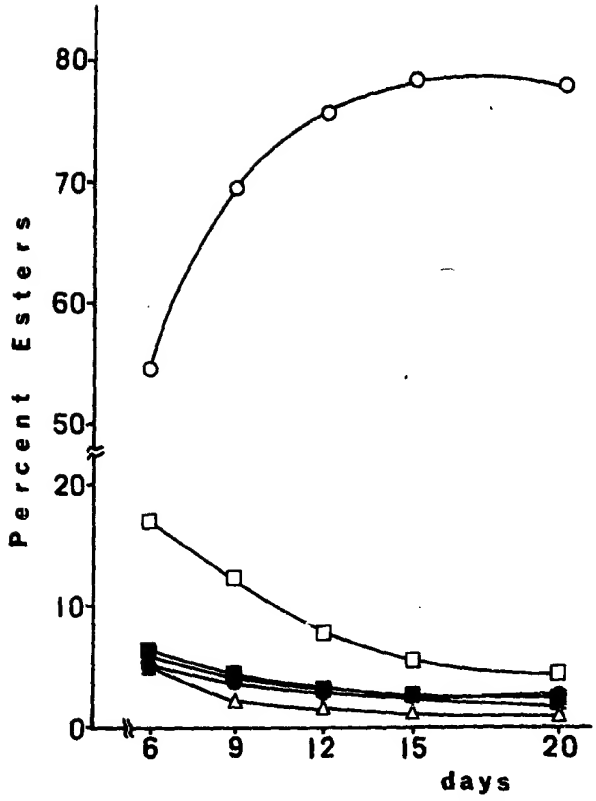

FIG. 2. Changes of methyl esters obtained from *Mortierella alpina* IFO 8568 grown on malt agar plates at 25 C. Palmitate (□), stearate (■), oleate (△), linoleate (▲), γ-linolenate (●) and arachidonate (○).

acid was less than 1%. As is shown in Figure 1, the weight of fatty acid methyl esters excluding arachidonate was constant, but the fatty acid compositions were changing markedly. This may suggest that the metabolic pathway from C16:0 → C18:0 → C18:1 → C18:2 → γ-C18:3 → C20:3(dihomo-γ-linolenic acid) → C20:4 exists also in the fungus. Dihomo-γ-linolenic acid, a precursor of arachidonic acid, amounted to only a small percentage of the total fatty acids, while the content of γ-linolenic acid was a few percent. It appears that younger cells contain higher percentages of saturated fatty acid, and older cells accumulate arachidonic acid.

Arachidonic acid content in the total fatty acids of the four strains of *Mortierella* was 68.5–78.8%, the highest value of which was 2.9 times higher than that from *E. exitialis* (3), the highest source previously known. The arachidonic acid content per dry cell weight was 22.2–26.4%, which was more than 20 times higher than the value reported by Iizuka (2) and three times higher than that reported by Ahern (5,6). The other four strains we examined gave similar values, but other *M. alpina* strains, such as ATCC 8979 and ATCC 36965, did not show exceptional arachidonic acid production when cultured on the malt agar medium.

Experiments with *M. alpina* IFO 8568 showed that similar percentages of arachidonic acid were contained in the fatty acids of neutral and polar lipid fractions, although the amount of total fatty acids from neutral lipids was about five times greater than that from polar lipids.

Methyl arachidonate was identified as all *cis* methyl eicosa-5,8,11,14-tetraenoate using the following criteria:

Elemental analysis. Found: C:79.34%, H:11.21%. Calcd: C:79.15%, H:10.77%.

Gas chromatography. Retention times of the sample on DEGS 15% (column temperature 190 C), SE-30 (column temperature 170 C) and OV-101 (column temperature 170 C) coincided with those f an authentic standard.

Gas chromatography-mass spectrometry. The mass fragmentation pattern obtained by separating the sample on DEGS 10% (column temperature 200 C) and ionizing at 70 eV corresponded closely to that of the authentic standard. The parent peak appeared at m/e 318.

^{1}H-NMR spectrometry. The ^{1}H-NMR spectrum for the sample resembled that of the standard. Taking the three methyl protons of the methyl ester group at 3.6 parts per million (ppm) as standard, there were eight protons (5.0–5.7 ppm) associated with carbon-carbon double bonds and six protons (2.6–3.3 ppm) with methylene groups between double bonds consistent with the structure of a methylene-interrupted tetraenoate. Signals derived from *trans* double bond protons were not detected.

^{13}C-NMR spectrometry. The spectra showed signals at 13.8, 22.4, 24.7, 25.5, 26.4, 27.0, 29.2, 31.4 and 33.0 ppm due to methylene carbon, at 51.2 ppm for the ester methyl carbons, and at 127.0, 127.3, 127.6, 127.9, 128.3 and 129.8 ppm for the olefinic carbons. Signals due to carbons of *trans* double bond were not detected. The spectrum was identical to that of the authentic standard.

In conclusion, we found that mycelia of *M. alpina*, which were incubated on malt agar plates at 25 C for 15 days, produced lipids, the fatty acid of which contained up to 78.8% of arachidonic acid. The arachidonic acid amounted to about 25% of the total dry cell weight. This permits easy isolation and purification of arachidonic acid.

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ERRATUM

In the paper "Cyclic Fatty Esters: Hydroperoxides from Autoxidation of Methyl 9-(6-Propyl-3-Cyclohexenyl)-(Z)8-Nonenoate" by R. A. Awl, E. N. Frankel, and D. Weisleder, Vol. 22, No. 10, pp. 721–730, there was an error in a figure legend.

The legend for Figure 6 should have read:

FIG. 6. Capillary gas chromatography-mass spectrometry chromatogram of hydroperoxide fraction of autoxidized cyclic ester I after hydrogenation and silylation.

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Inhibitory Effect of Curcumin on Fatty Acid Desaturati n in *Mortierella alpina* 1S-4 and Rat Liver Micros mes

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An extract of rhizomes of *Curcuma longta* L. (turmeric) inhibited the desaturation of dihomo-γ-linolenic acid (DGLA) in the arachidonic acid (AA) producing fungus *Mortierella alpina* 1S-4. The factor responsible for this phenomenon was isolated and identified as curcumin (diferuloyl methane). Mycelial DGLA levels increased about two-fold (22.3 mg/g dry weight) with a concomitant decrease in AA levels when the fungus was cultivated with curcumin. The 50% inhibitory concentration against Δ5 desaturase was 27.2 μM. Curcumin also inhibited rat liver microsomal Δ5 and Δ6 desaturases.
Lipids 27, 509–512 (1992).

Polyunsaturated fatty acids (PUFA), especially those of the C_{20} series, such as dihomo-γ-linolenic acid (8,11,14-*cis,cis,cis*-eicosatrienoic acid, DGLA), arachidonic acid (5,8,11,14-*cis,cis,cis,cis*-eicosatetraenoic acid, AA) and 5,8,11,14,17-*cis,cis,cis,cis,cis*-eicosapentaenoic acid, are not only important precursors of prostaglandins, but they also regulate membrane fluidity as primary components of biological membranes and affect various physiological activities. In higher animals, PUFA of the ω6 and ω3 series cannot be synthesized unless linoleic acid (ω6) or α-linolenic acid (ω3) is supplied in the diet. Thus, compounds that regulate the metabolism of these PUFA are of profound interest, as they may not only serve as important means of clarifying the physiological significance of PUFA biosynthesis, but they also may be useful as new types of drugs.

We have reported previously (1,2) that sesamin and other lignan compounds found in sesame seeds inhibit the conversion of DGLA to AA (Δ5 desaturation). In the present study we tested several extracts of crude drugs and spices for their effects on the fatty acid composition of the AA producing fungus, *Mortierella alpina* 1S-4. We found that the DGLA/AA ratio in the fungus increased significantly when grown in a medium supplemented with an ethanol-extract of turmeric. In this paper we report the isolation and characterization of the inhibitory constituent responsible for this phenomenon and its effect on the desaturation system of this fungus, as well as on rat liver microsomal desaturases.

MATERIALS AND METHODS

Chemicals. Curcumin was purchased from Wako Pure Chemicals (Osaka, Japan). Turmeric, other spices and crude drugs were obtained from a local market. Radiolabeled fatty acids were obtained from Amersham International (Buckinghamshire, U.K.). All other reagents were of analytical grade.

Microorganism and cultivation. Mortierella alpina 1S-4 (3,4) was inoculated as a spore suspension into a 10-mL flask containing 2 mL of medium A (2% glucose and 1% yeast extract, pH 6.0) or medium B (4% glucose and 1% yeast extract, pH 6.0) and then incubated at 28°C with reciprocal shaking at 120 strokes/min, unless otherwise noted. When crude drugs and spices were tested for their effects on the fatty acid composition of the fungus, 0.5 g of each test substance was first extracted twice with 5 mL of ethanol. The extract was evaporated to dryness and then redissolved in a small volume of ethanol. The extracts were mixed with sterile medium A at concentrations of 0.1–1% (w/vol) when the fungus was inoculated and grown as described above. The ethanol concentrations were usually less than 5%.

Fatty acid analysis. Mycelial cells were harvested by suction filtration, washed with distilled water and dried at 100°C overnight for subsequent fatty acid analysis by gas-liquid chromatography (GLC) after transmethylation with methanolic HCl as detailed elsewhere (3,5).

Cell-free extract. Washed mycelia (*ca.* 7 g) of *M. alpina* 1S-4 were suspended in 1 mL of 50 mM potassium phosphate buffer, pH 7.4, containing 1 mM dithiothreitol and ground in an ice-cooled mortar with 2 g of sea sand for 50 min. One mL of buffer was added 10, 20, 30 and 40 min later. After centrifugation (10,000 × *g*, 4°C, 30 min), the supernatant was used as the enzyme solution.

Preparation of rat liver microsomes. Microsomes were prepared from male Wistar rats maintained on a pellet diet for 8 wk as described previously (2).

Assay of desaturase activities. The cell-free extract of *M. alpina* 1S-4 was assayed for Δ5 desaturase activity by using radiolabeled DGLA as previously described (2). Rat liver microsomal Δ5, Δ6 and Δ9 desaturase activities were measured by the method of Svensson (6) using radiolabelled DGLA, linoleic acid and stearic acid, respectively, as substrates.

Other methods. Protein concentrations were determined by the method of Bradford (7). Mass spectra were recorded on a Hitachi (Tokyo, Japan) M-80B at 70 eV. ^1H nuclear magnetic resonance (NMR) spectra were measured with Nicolet (Fermont, CA) NT-360 using CD_3OD as solvent and tetramethylsilane as internal standard.

RESULTS

Screening of Δ5 desaturation inhibitors. The effects of 62 crude drugs and 67 spices on the fatty acid profile of *M. alpina* 1S-4 were tested. The effects of some crude drugs and spices (which are commonly known and easily obtainable in Japan) on DGLA and AA production of the fungus are given in Table 1. Considering the DGLA/AA ratios of the fungus grown in the presence of the extracts as an index for the estimati n of Δ5 desaturation inhibitory activity, we found that the DGLA/AA ratio of the fungus cultured with turmeric extract was 0.43. This

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Abbreviations: AA, arachidonic acid; DGLA, dihomo-γ-linolenic acid; FA, fatty acid; GLC, gas-liquid chromatography; NMR, nuclear magnetic resonance; PUFA, polyunsaturated fatty acid; TLC, thin-layer chromatography.

TABLE 1

Effects of Extracts of Spices and Crude Drugs on the DGLA and AA Production of *M. alpina* 1S-4[a]

Spice	Growth (mg/mL)	FA (mg/mL) DGLA	FA (mg/mL) AA	DGLA/AA ratio
Pepper	11.4	0.38	1.48	0.26
Cinnamon	10.5	0.25	1.07	0.23
Parsley	8.4	0.10	0.35	0.29
Turmeric	9.1	0.17	0.40	0.43
Nutmeg	10.4	0.19	0.77	0.25
Tarragon	9.4	0.14	0.52	0.27
Marjoram	10.1	0.19	0.74	0.26
Coriander	9.8	0.20	0.87	0.23
Star anise	9.2	0.16	0.73	0.22
Ajowan	9.1	0.13	0.65	0.20
Mustard	9.0	0.12	0.48	0.25
Rosemary	7.1	0.08	0.40	0.20
Cumin	9.9	0.04	0.14	0.29
Thyme	10.7	0.29	1.24	0.23
Cardamom	9.9	0.17	0.73	0.23
Tochuu[b]	9.5	0.11	0.38	0.29
Nyotei[c]	7.7	0.09	0.33	0.27
Ireisen[d]	11.3	0.18	0.67	0.27
Bouhuu[e]	8.6	0.07	0.24	0.29
Goboushi[f]	11.4	0.16	0.59	0.27
Control	11.9	0.25	1.20	0.21

[a] *M. alpina* 1S-4 was grown in medium A supplemented with the extracts of the materials given in the Table. The concentrations of the extracts added were 0.1% (w/vol), and the other conditions were as described in Materials and Methods. All values are means of two determinations.

[b] Bark of *Eucommia ulmoides* Oliver.

[c] Fruit of *Liguotrum japonicum* Thunb.

[d] Root of *Clematis terniflora* DC.

[e] Root of *Seseli libanotis* Koch var. *daucifolia* DC.

[f] Fruit of *Arctium lappa* L.

was about double that of the control (0.21). However, those cultured with the other extracts (including those not shown in Table 1) ranged from 0.20 to 0.29. The extracts which resulted in DGLA/AA ratio higher than 0.27 (or 1.3-fold that the control) were tested again as to their dose-dependent effects on the DGLA/AA ratio, but none of them except for turmeric extract was found to be positive (data not shown). As shown in Table 2, the presence of turmeric extract of up to 20 μL in the culture medium did not affect fungal growth, but some inhibitory effect was observed with 50 μL. The levels of palmitic acid, oleic acid and DGLA appeared to increase to some extent, whereas those of AA decreased markedly. Mycelial DGLA concentration reached 28.7 mg/g dry mycelia, a value approximately 1.7 times that of the control (no supplement).

Isolation and identification of the inhibitory component. In order to characterize the active compound(s) responsible for this inhibition, we eluted the ethanol extract through silica gel with successive solvent systems of *n*-hexane/ethyl acetate (95:5 and 90:10, vol/vol) and methanol. Based on the elution profile at 254 nm, the extract was separated into eight fractions. The inhibitory effects of each fraction were monitored by growing the fungus in medium B supplemented with each fraction (0.01–0.1% w/vol culture broth), followed by analysis of the fatty acid profile. The fungus grown in the presence of the methanol-

TABLE 2

Effect of an Ethanol Extract of Turmeric on the Fatty Acid Profiles of *M. alpina* 1S-4[a]

Volume added (μL)[b]	Fatty acid composition (wt%) 0	5	10	20	50
16:0	13.9	20.4	18.8	22.0	25.2
18:0	8.2	8.6	8.5	7.6	7.5
18:1	7.9	15.7	16.4	20.4	25.0
18:2	6.5	8.5	8.2	9.3	9.3
18:3ω6	4.0	2.8	3.3	3.2	3.4
DGLA	4.3	6.2	6.2	7.2	10.5
AA	55.2	37.8	38.6	30.3	19.1
DGLA (mg/g)	16.9	22.0	25.7	26.5	28.7
AA (mg/g)	217.3	133.9	160.2	111.5	52.2
Total FA (mg/g)	393.7	354.2	415.1	368.0	273.3
Growth (mg/mL)	17.6	19.9	17.4	17.6	12.1
DGLA/AA ratio	0.08	0.16	0.16	0.24	0.55

[a] *M. alpina* 1S-4 was grown in medium B supplemented with the turmeric extract at 28°C for 7 d. All values are means of two determinations.

[b] The ethanol extract obtained from 0.5 g of turmeric was resuspended in 4 mL ethanol, then the indicated volumes were added to 2 mL of sterile medium A.

luted fraction accumulated about twice as much DGLA (27.9 mg/g dry mycelia) than did controls (14.7 mg/g dry mycelia). DGLA concentrations were about 12–14 mg/g dry mycelia in fungi grown with the other fractions. This fraction was separated into several bands in reverse phase thin-layer chromatography (TLC, $KC_{18}F$; Whatman, Fairfield, NJ) using 70% methanol as the developing solvent. Therefore, this fraction was subfractionated on a reverse phase column packed with octadecylsilane-bonded silica gel (Nakarai Chemicals, Kyoto, Japan) using various concentrations of aqueous methanol as eluent. Seven subfractions were obtained which were separately tested for their effects on the fatty acid profile of the fungus as described above. One fraction, comprised of a yellow pigment, showed a similar effect to that found in the crude extract. The DGLA/AA ratio of the fungus grown with this fraction was 0.26, and that of the control was 0.13. On the other hand, the DGLA/AA ratios of fungi grown with the other fractions were 0.12–0.16. The yellow fraction was homogeneous in reverse phase TLC as judged by UV detection (254 nm). Mass spectra showed the molecular ion at *m/z* 368 (relative intensity 27%); the other major fragmentation ion peaks were 350 (28), 338 (3), 320 (5), 272 (9), 244 (6), 232 (12), 217 (17), 203 (5), 190 (42), 177 (100), 161 (8), 150 (26), 145 (34), 137 (50), 131 (27), 117 (22), 103 (15), 89 (27), 77 (23), 65 (13), 51 (13) and 39 (8). ^1H NMR showed signals (*δ*, ppm) at 3.91 (*s*, 6H, CH_3), 5.96 (*s*, 1H, C=C), 6.62 (*d*, 2H, C=C), 6.81 (*d*, 2H, Ar), 7.12 (*d*, 2H, Ar), 7.21 (*s*, 2H, Ar) and 7.57 (*d*, 2H, C=C). These data agree well with those of authentic standard and they correspond to those of the enol form of curcumin, the structure of the keto form of which is shown in Figure 1.

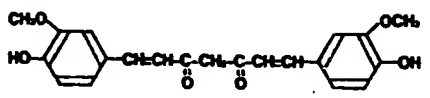

FIG. 1. Chemical structure of curcumin.

Effects of curcumin on DGLA production. Commercially obtained curcumin was dissolved in ethanol and added to sterilized medium B on which *M. alpina* 1S-4 was grown at 28°C for one week. As shown in Figure 2, the production of DGLA increased dose-dependently. The DGLA production rate was about 0.42 mg/mL culture broth per 0.1 mg/mL of curcumin. A higher concentration of curcumin inhibited fungal growth (data not shown).

Effect of curcumin on the conversion of DGLA to AA in vitro. The effect of curcumin on the conversion of DGLA to AA was investigated *in vitro* using a cell-free extract of the fungus. As shown in Figure 3a, increasing concentrations of curcumin markedly reduced the conversion of DGLA to AA. The 50% inhibitory concentration against Δ5 desaturase was 27.2 μM. On the other hand, the inhibition against Δ6 desaturase was less than 10% (data not shown). This result is consistent with the observation that the mycelial DGLA level of the fungus increased with a concomitant decrease in AA on growth in the presence of curcumin.

Inhibition effect in rat liver microsomes. Since curcumin inhibited Δ5 desaturation in the fungus, we further tested its effect on the desaturation systems of animals using rat liver microsomes (Fig. 3b). Unlike sesamin, which inhibits Δ5 desaturation in a specific manner (2), curcumin not only inhibited Δ5 desaturation, but the conversion of linoleic acid to γ-linolenic acid, *i.e.*, Δ6 desaturation. In the presence of 80 μM curcumin, the inhibition of Δ5 and Δ6 desaturation was 49% and 18%, respectively.

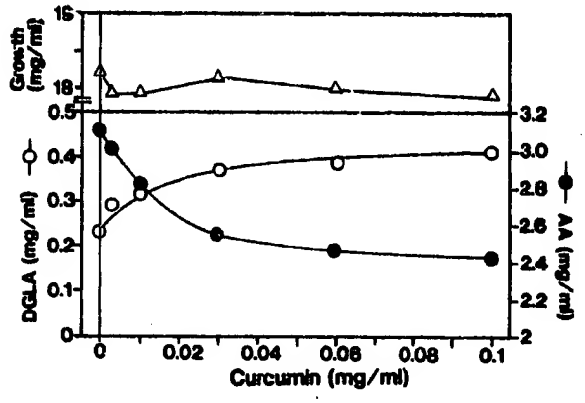

FIG. 2. Effects of curcumin on the production of DGLA and AA by *M. alpina* 1S-4. Curcumin was added as an ethanol solution to sterile medium B at the final concentrations indicated, then *M. alpina* 1S-4 was cultured at 28°C for one week. All value are means of three determinations, the standard deviation being less than 10%.

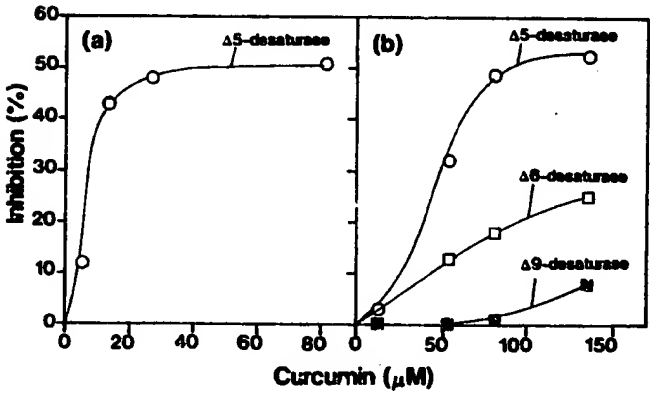

FIG. 3. Effects of curcumin on the Δ5 desaturase activities in cell-free extract of *M. alpina* 1S-4 (a) and rat liver microsomes (b). The reaction was done at 28°C (a) or 37°C (b), using the respective labeled fatty acids as substrate under the conditions described previously (2). The percentage of inhibition was calculated from the amount of radiolabeled fatty acids formed as compared with the control (no curcumin addition). The specific activity of Δ5 desaturase in the fungal cell-free extract was 24 pmol/min/mg protein, and those of rat microsomal Δ5, Δ6 and Δ9 desaturases were 131, 19 and 38 pmol/min/mg protein, respectively. All values are means of three determinations, the standard deviation being less than 10%.

DISCUSSION

These studies were undertaken to screen for Δ5 desaturation inhibitors from natural sources. Since the fatty acid composition can be expected to reflect desaturation activity in cells, we performed the screening by growing a potent AA producer, *M. alpina* 1S-4, in the presence of the test materials, followed by analysis of the fungal fatty acids. We found that curcumin, the major component of turmeric (*Curcuma longta*, L.) (8), mainly inhibited Δ5 desaturation in *M. alpina* 1S-4 fungus. However, in rat liver microsomes, curcumin markedly inhibited Δ6 desaturation. Curcumin has been studied intensively by many investigators and has been shown to have inflammatory activity (9–17), as well as showing inhibitory effects on mammalian 5-lipoxygenase and cyclooxygenase (18). However, as far as we know, there has been no report on its effect on the fatty acid desaturation system.

Except for our earlier paper (2), there has been no report demonstrating the occurrence of desaturase inhibitors in nature. Some unnatural desaturase inhibitors have been synthesized, and most of them are analogs of fatty acids which nonspecifically inhibit any of the desaturases involved in PUFA biosynthesis. For example, several *trans*-octadecenoic acids are competitive inhibitors of Δ9, Δ6 and Δ5 desaturases of rat liver microsomes (19,20). A kinetic study of the inhibition mode of curcumin was not done, but the inhibition is probably not competitive. On a structural basis, curcumin should not compete with any fatty acid for any desaturase.

The enzyme Δ5 desaturase converts DGLA to AA with the result that a precursor for the 1-series prostaglandins is converted to a precursor for the 2-series. Since curcumin inhibited the Δ5 desaturase, it may be able to influence the balance of the two fatty acids and, therefore, alter various metabolic pathways. It would be of interest to investigate the effect of curcumin on the fatty acid desaturation (or biosynthesis) in humans, since curcumin is a component of an edible spice that is widely consumed.

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Bajpai et al., "Effects of Aging Mortierella Mycelium on Production of Arachidonic and Eicosapentaenoic Acids", pp. 775-780, 1991, JAOCS, vol. 68, Oct.

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Production of arachidonic acid by *Mortierella* fungi

Selection of a potent producer and optimization of culture conditions for large-scale production

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Summary. Various *Mortierella* fungi were assayed for their productivity of arachidonic acid (ARA). Only strains belonging to the subgenus *Mortierella* accumulated detectable amounts of ARA together with dihomo-γ-linolenic acid. None of the strains belonging to the subgenus *Micromucor* tested accumulated these C-20 fatty acids, although they produced a C-18 fatty acid, γ-linolenic acid. A soil isolate, *M. alpina* 1S-4, was found to grow well in a liquid medium containing glucose and yeast extract as carbon and nitrogen sources, respectively. Addition of several natural oils such as olive and soybean oils to the medium increased the accumulation of ARA. Under optimal culture conditions in a 5-l bench-scale fermentor, the fungus produced 3.6 g/l of ARA in 7 days. On cultivation for 10 days at 28° C in a 2000-l fermentor, the same fungus produced 22.5 kg/kl mycelia (dry weight) containing 9.9 kg lipids, in which ARA comprised 31.0% of the total fatty acids. On standing the harvested mycelia for a further 6 days, major mycelial fatty acids (i.e. palmitic acid, oleic acid, linoleic acid, etc.) other than ARA rapidly decomposed and the ARA content of the total fatty acids reached nearly 70%.

Introduction

5,8,11,14-*cis*-Eicosatetraenoic acid (arachidonic acid, ARA) is a C-20 polyunsaturated fatty acid (PUFA), known to be a biogeneic precursor of prostaglandins and leukotrienes (Marx 1982). In addition, it has recently attracted great interest due to its several unique biological activities (Das et al. 1987; Horrobin and Huang 1987). At present, the oil extracted from porcine liver is generally used as the main source of ARA. However, the ARA content of this oil is so low that it is not a practical source of this fatty acid. There have been a few papers reporting that microorganisms produce ARA as a minor component of cellular lipids (Haskins et al. 1963; Tyrrell 1966; Iizuka et al. 1979). However, no attention had been paid to microorganisms as practical sources of ARA before our recent studies (Yamada et al. 1987a, b).

In previous papers, we reported that isolated filamentous fungi, *Mortierella elongata* 1S-5 and several strains of *M. alpina* are potent producers of ARA and other C-20 PUFAs, i.e. eicosapentaenoic acid (EPA) and dihomo-γ-linolenic acid (Yamada et al. 1987a, b; Shimizu et al. 1988a, b). Subsequently, Totani and Oba (1987) reported that a strain of *M. alpina* accumulates ARA when cultivated statically on solid medium for a long time. Because liquid culture is more favorable for large-scale production of ARA during a short culture period, we have continued further screening studies to obtain suitable microorganisms for ARA production in liquid media. In this work, the potential of several ARA-producing *Mortierella* fungi as practical producers of ARA, and the culture conditions for large-scale fermentation under which maximum ARA productivity can be obtained were studied.

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Materials and methods

Chemicals. Oils were obtained from Yamakei Sangyo (Osaka, Japan), Wako Pure Chemicals (Osaka, Japan), Nakarai Chem-

icals (Kyoto, Japan) and Sigma (St. Louis, Mo, USA). All other reagents used in this work were as described previously (Yamada et al. 1987a; Shimizu et al. 1988a).

Microorganisms. *Mortierella elongata* 1S-5 (AKU 3999), *M. alpina* 1S-4 (AKU 3998) and *M. alpina* 20-17 (AKU 3996) were isolated by us from natural sources as potent ARA producers (Yamada et al. 1987a); *M. alpina* 1-83 (AKU 3995) was newly isolated from a soil in Kyoto Prefecture. All other *Mortierella* strains were from our stock culture (AKU culture collection, Faculty of Agriculture, Kyoto University).

Media and cultivation. Medium YM and medium GY were as described previously (Yamada et al. 1987a; Shimizu et al. 1988b). Medium G and medium O were medium YM supplemented with 9% glucose and 1% olive oil, rspectively. Cultivations were carried out at 28° C as described previously (Shimizu et al. 1988b) unless otherwise stated. Other conditions are given in the legends to the respective figures and tables.

Analyses. Extraction and determination of fatty acids and other analyses were carried out as described previously (Shimizu et al. 1988b).

Results

Comparison of mycelial fatty acid compositions in various Mortierella strains

Ten *Mortierella* strains including those of two different subgenera, *Mortierella* and *Micromucor*, were grown at either 12° C or 28° C and then assayed for their mycelial fatty acid profiles (Table 1). A C-18 PUFA, γ-linolenic acid, was found as one of the major mycelial fatty acids in every strain tested regardless of subgenera. However, strains belonging to the subgenus *Mortierella* were quite different from those belonging to the subgenus *Micromucor* in that the former strains could accumulate C-20 PUFAs (i.e. dihomo-γ-linolenic acid and ARA at 28° C and EPA in addition t those at 12° C). This result agrees well with our previous observation that all the EPA-producing

Table 1. Mycelial polyunsaturated fatty acid profiles of various strains belonging to the genus *Mortierella*[a]

Subgenus	Growth temp. (° C)	% in total FA				
		GLA[b] 18:3	DGLA[b] 20:3	ARA[b] 20:4	EPA[b] 20:5	Total PUFA[b,c]
Mortierella						
M. bainieri	12	3.8	5.5	19.4	4.1	32.8
IFO 8569	28	5.8	5.4	21.5	0	32.7
M. verticillata	12	5.8	6.4	18.0	7.7	37.9
IFO 8575	28	6.0	4.8	25.7	0	36.5
M. globalpina	12	4.9	6.4	20.9	2.7	34.9
CBS 360.70	28	5.1	3.4	16.2	0	24.7
M. alpina[d]	12	5.3	8.9	15.6	2.0	31.8
CBS 210.32	28	3.6	1.8	6.9	0	12.3
M. zychae	12	5.0	5.9	15.1	2.7	28.7
CBS 316.52	28	6.0	3.3	16.3	0	25.6
Micromucor						
M. ramanniana[e]	12	19.2	0	0	0	19.2
IFO 8187	28	14.8	0	0	0	14.8
M. isabellina	12	21.8	0	0	0	21.8
IFO 6739	28	16.5	0	0	0	16.5
M. vinacea	12	14.1	0	0	0	14.1
IFO 7875	28	13.0	0	0	0	13.0
M. humicola	12	15.2	0	0	0	15.2
IFO 8289	28	13.6	0	0	0	13.6
M. nana	12	13.5	0	0	0	13.5
IFO 8794	28	8.0	0	0	0	8.0

[a] Each strain was cultivated in medium YM for 7 days under the conditions given in Materials and methods

[b] Abbreviations used: FA, fatty acid; GLA, 18:3, γ-linolenic acid; DGLA, 20:3, dihomo-γ-linolenic acid; ARA, 20:4, arachidonic acid; EPA, 20:5, eicosapentaenoic acid; PUFA, polyunsaturated fatty acid

[c] Total PUFA: sum of GLA, DGLA, ARA and EPA

[d] *M. alpina* var. *renispora* CBS 210.32

[e] *M. ramanniana* var. *angulispora* IFO 8187

Mortierella strains rep rted previ usly (Shimizu et al. 1988a, b) also bel ng to the subgenus *Mortierella*.

Screening for strains of the subgenus Mortierella showing high ARA productivity

Based on the results of Table 1, we assayed various strains belonging to the subgenus *Mortierella* for their productivities of ARA in two kinds of growth media, medium G and medium O (Fig. 1). Four strains of *M. alpina*, 1S-4, 20-17, 1-83 and CBS 210.32, accumulated about 0.4 mg/ml of ARA in medium O. In *M. alpina* strains 1S-4, 20-17 and 1-83, the amounts of ARA accumulated were essentially the same when grown with high concentration of glucose (medium G). On the other hand, *M. alpina* CBS 210.32 produced 0.86 mg/ml ARA when grown with medium G. *Mortierella rishikesha* and *M. kuhlmanii* also showed high production of ARA in medium G (0.62 and 0.70 mg/ml, respectively), but the amounts of ARA in medium O were small. Medium GY was also suitable for ARA production by these four *M. alpina* strains (data not shown), and they accumulated this fatty acid as the most predominant mycelial fatty acid (30%–50%, by weight, in total extractable mycelial fatty acids). Based on these results we selected these four *M. alpina* strains for the following studies.

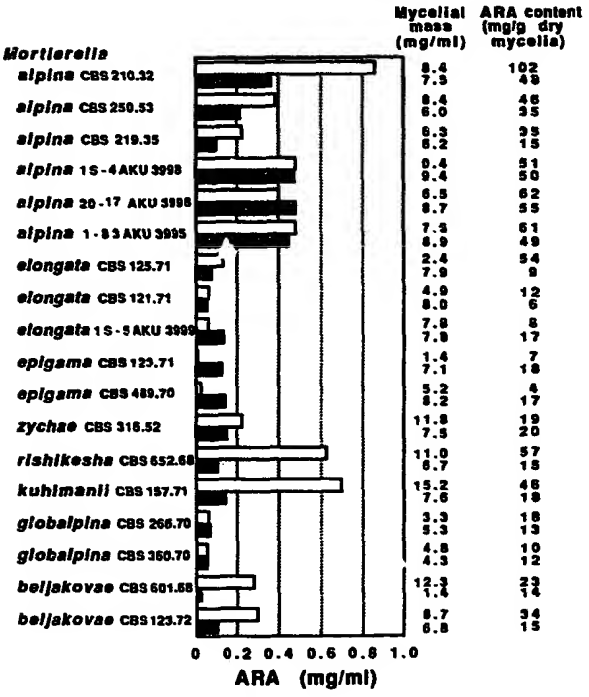

Fig. 1. Arachidonic acid (ARA) production by various *Mortierella* strains belonging to the subgenus *Mortierella*. Each strain was cultivated in test tubes (25 × 200 mm) containing 10 ml medium G *(open bars)* or medium O *(meshed bars)* at 28° C with reciprocal shaking (300 strokes/min) for 7 days

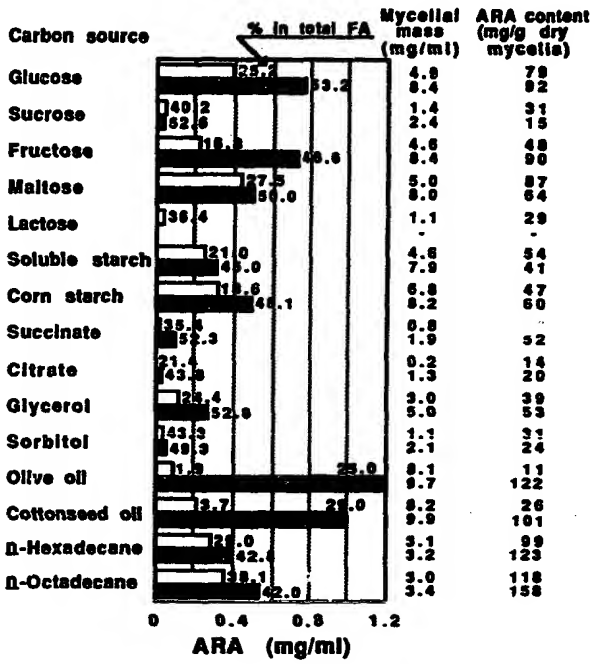

Fig. 2. Effects of carbon source on the production of ARA by *M. alpina* 1S-4 cultivated with basal medium composed of 1% corn steep liquor, 0.05% yeast extract *(open bars)* or 1% yeast extract *(meshed bars)* for 6 days with addition of each of the indicated carbon sources (2%) under the conditions given in Materials and methods. The pH of the media was adjusted to 6.0 before inoculation. FA, fatty acid

Factors affecting ARA production

The carbon compounds listed in Fig. 2 were tested as carbon sources for the growth of *M. alpina* 1S-4 and its ARA production in media containing either corn steep liquor or yeast extract as major nitrogen sources. It grew rapidly in media containing glucose regardless of the nitrogen source used and yielded 5–10 mg/ml of mycelia after 6 days. Fructose, maltose, soluble starch and corn starch also gave almost the same mycelial yields and ARA contents to those with glucose. The ARA contents of the mycelia grown with *n*-octadecane r *n*-hexadecane were 1.2–1.5-fold higher than th se with glucose, but the mycelial

yields were poor. The fungus showed high pr - duction of ARA when grown with live oil r cottonseed oil as a carbon source and 1% yeast extract as a nitrogen source. The ptimum concentration of glucose at a fixed concentrati n f yeast extract (1%) was 2%–4%, with which ARA production reached 0.8–1.0 mg/ml (90–100 mg/g dry mycelia) after 5 days incubation at 28° C. With corn steep liquor as the nitrogen source (1%), the optimal concentration of glucose was 2%, with which the fungus accumulated 0.65 mg/ml (85 mg/g dry mycelia). In this case, a further increase in the glucose concentration brought about a marked decrease in the mycelial ARA content (data not shown). Other *M. alpina* strains also utilized favourably these five carbohydrates and yielded enough mycelia (7–10 mg/ml) rich in ARA (40–90 mg/g dry mycelia) under the same test conditions as given in the legend to Fig. 2 (data not shown).

The effects of nitrogen source on production of ARA by *M. alpina* 1S-4 were examined by adding various kinds of organic or inorganic nitrogen sources (final concentration, 1%) to a medium containing 2% glucose and 0.05% yeast extract. Yeast extract, soybean meal and corn steep liquor were found to be suitable nitrogen sources for ARA production. The optimum concentration of yeast extract at fixed concentrations of glucose (2% and 4%) was 1%. Further increase in yeast extract concentration resulted in a marked decrease in ARA production, although mycelial yields were higher than those with 1% yeast extract (data not shown).

The amount of ARA produced by *M. alpina* 1S-4 increased with mycelial growth, reaching the maximum (0.88 mg/ml; 96 mg/g dry mycelia) at 28° C after incubation f r 6 days in medium GY. Cultivati n at higher temperatures (30°–35° C) resulted in decreased accumulati n of t tal lipids in mycelia, alth ugh mycelial growth was still rapid and dense. The ARA content f the lipids als markedly decreased and oleic acid was found t be the most predominant mycelial fatty acid at these temperatures. For *M. alpina* 1-83 and *M. alpina* CBS 210.32, the maximum values (0.73 mg/ml or 83 mg/mg dry mycelia and 0.81 mg/ml or 91 mg/g dry mycelia, respectively) were also attained at 28° C, whereas the maximum value (0.84 mg/ml; 98 mg/g dry mycelia) with *M. alpina* 20-17 was obtained at 22° C.

A suitable initial pH of medium GY for ARA production and growth was found to be between 5 and 7 in every strain tested.

Effects of various oils on the production of ARA

Since the mycelial ARA content of *M. alpina* 1S-4 significantly increased on incubation with *n*- c tadecane, and olive oil and cottonseed oil were utilized well as carbon sources for fungal growth and ARA production (see Figs. 1, 2), the fungus was grown in medium GY supplemented with various fatty acids, *n*-alkanes or natural oils (final concentration, 1%) for 7 days at 28° C. Marked stimulation of ARA production (3.6 mg/ml) was observed when the fungus was grown with soybean oil, which was about 2.8-fold higher than that without oil. Corn (ARA production, 1.9 mg/ml), olive (1.8 mg/ml), peanut (1.8 mg/ml), rapeseed (1.8 mg/ml) and fish (1.8 mg/ml) oils also showed stimulatory effects.

Table 2. Bench-scale production of ARA by several *M. alpina* strains under optimal culture conditions

M. alpina strains	Culture conditions[a]				Productivity				
	Total glucose used (g/l)	Temp. (° C)	pH	Culture period (days)	Mycelial mass (g/l)	Total FA[b] (mg/g dry mycelia)	ARA content (% in total FA)	ARA yield (mg/g dry mycelia)	(g/l)
CBS 210.32	156	28	5.5	5	21.0	186	28	52.4	1.1
1S-4 AKU 3998	50	24	6.0	7	24.5	420	35	147.0	3.6
	104	28	6.0	10	22.0	440	31	136.4	3.0
20-17 AKU 3996	20	20	5.5	5	10.0	485	52	252.5	2.5
1-83 AKU 3995	100	28	6.0	6	18.5	395	39	154.1	2.9

[a] Each strain was cultivated in a 5-l bench-scale fermentor (Mitsuwa Rika, Osaka, Japan) with 2.5 l medium GY. Glucose was periodically added to maintain a 1.5%–2.5% concentration except for the case of *M. alpina* 20-17. All cultivations were carried out with aeration at 1 vvm and agitation at 400 rpm under the conditions given in the table

[b] FA, fatty acid

Large-scale production of ARA under optimal culture conditions

From the results of experiments on individual and combined factors affecting ARA production described above, the optimal culture conditions for each *M. alpina* strain in a 5-l bench-scale fermentor were determined as shown in Table 2. All of the strains produced more than 1 g/l ARA under these conditions but *M. alpina* 1S-4 was selected for further large-scale production because it showed the highest accumulation of ARA. On cultivation for 10 days in a 2000-l fermentor under the conditions given in the legend to Fig. 3, the fungus produced 22.5 kg/kl mycelia (dry weight) containing 44.0% of lipids, in which ARA comprised 31.0% of the total fatty acids. This value corresponded to 3 kg/kl of ARA (Fig. 3a). When the harvested mycelia were allowed to stand for a further few days at room temperature, the palmitic acid, stearic acid, oleic acid and linoleic acid in the mycelia were rapidly consumed. On the other hand, the ARA accumulated in the mycelia was unchanged and, as a result, the specific mycelial ARA content increased. For example, it reached 67.4% of the total fatty acids on standing for a further 6 days at 28° C, as shown in Fig. 3b. A similar increase in C-20 PUFA content was also observed in the case of EPA production by *Mortierella* fungi (Shimizu et al. 1988c).

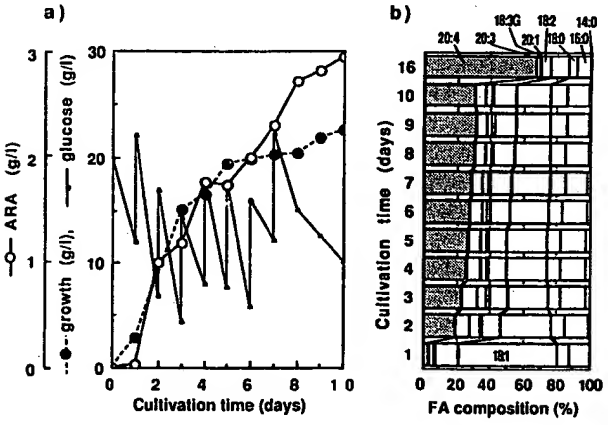

Fig. 3a, b. Time course of the production of ARA by *M. alpina* 1S-4 cultivated in a 2000-l fermentor (Kansai Chemical Engineering, Co., Osaka, Japan) containing 1400 l medium composed of 2% glucose, 1% yeast extract and 0.2% olive oil, pH 6.0. Cultivation was carried out at 28° C with aeration at 1 vvm and agitation at 80 rpm. **a.** Glucose was added periodically to the medium as shown. **b.** Changes in the mycelial fatty acid composition during growth. The following abbreviations are used for the fatty acids: 14:0, myristic acid; 16:0, palmitic acid; 18:0, stearic acid; 18:1, leic acid; 18:2, linoleic acid; 18:3G, γ-linolenic acid; 20:1, eicosenoic acid; 20:3, dihomo-γ-linolenic acid; 20:4, ARA; FA, fatty acid

Discussion

We have reported that many fungi belonging to the genus *Mortierella* produce large amounts of C-20 PUFAs of the n-6 family (i.e. dihomo-γ-linolenic acid and ARA) together with a C-18 PUFA of the same family (i.e. γ-linolenic acid) when grown with glucose as the major carbon source and that the resultant mycelia are rich sources of these PUFAs (Yamada et al. 1987a, b; Shimizu et al. 1988a, b). The data in Table 1 provide further evidence for this finding. However, the data clearly show that some of them do not accumulate C-20 PUFAs; the strains belonging to the subgenus *Micromucor* seemed to lack the ability to produce C-20 PUFAs, although they were excellent in producing γ-linolenic acid. Conversely, all the strains belonging to the subgenus *Mortierella* tested produced C-20 PUFAs as shown in Table 1 and Fig. 1. This characteristic difference in mycelial fatty acid profiles between these two subgenera may be useful not only to obtain potent C-20 PUFA producers but also to distinguish these subgenera from each other. In addition, lowering the growth temperature led to the additional accumulation of a new C-20 PUFA of the n-3 family, EPA, in every ARA producer tested, suggesting that formation of C-20 PUFAs of the n-6 family is necessary for EPA production. This observation agrees well with our previous observation that the enzymatic synthesis of EPA with cell-free extracts of *M. alpina* 1S-4 requires lipids containing C-20 PUFAs of the n-6 family (Shimizu et al. 1988a).

Mortierella alpina 1S-4 and four other *M. alpina* strains selected here for large-scale production of ARA can grow rapidly with a variety of carbon sources, such as glucose, maltose, starch, *n*-octadecane and several plant and animal oils, and produce mycelia rich in ARA. They show high ARA productivities even when grown with only 2% glucose, while the other strains require higher concentrations of glucose (ca. 10%) for high ARA accumulation (Fig. 1). These characteristics indicate that these *M. alpina* strains are more favourable than the other strains tested for practical purposes.

Another interesting finding is that several natural oils, when added to the growth medium, stimulated ARA production. All of the effective oils were found to contain oleic acid and linoleic

acid as major fatty acids. Probably a porti n f them were utilized as precursors for ARA synthesis, which might have resulted in increased mycelial ARA content.

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Bajpai et al., "Effects of Aging Mortierella Mycelium on Production of Arachidonic and Eicosapentaenoic Acids", pp. 775-780, 1991, JAOCS, vol. 68, Oct.

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Effects of Aging *Mortierella* Mycelium on Production of Arachidonic and Eicosapentaenoic Acids

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Arachidonic acid and eicosapentaenoic acid (EPA) are important intermediates of eicosanoid metabolism and are presently the subject of extensive nutritional and medical research. The effects of mycelial aging on production of these fatty acids were investigated as part of a research program directed toward examining the feasibility of economically producing these products by fungal fermentation.

Arachidonic acid content of *M. alpina* ATCC 32222 increased from 4.1–8.3% to 13–16% during aging while lipid content of mycelium increased from 14–18% to 33–45%. Maximum lipid content produced in biomass during storage declined as harvesting time was increased from 3 to 6 days while maximum arachidonic acid content in lipid increased. Maximum lipid and arachidonic acid was produced during aging at pH 8, whereas arachidonic acid content of lipids was highest in mycelium aged at pH 6. EPA content of *M. elongata* NRRL 5513 biomass increased during aging, reaching a maximum after 22–28 days. When the pH of the culture prior to harvesting was adjusted in the range of pH 4–9, pH values for development of maximum EPA in biomass and in lipids during storage were found to be 6 and 7, respectively. Temperature of aging had little effect on arachidonic acid or EPA content.

KEY WORDS: Arachidonic acid, eicosapentaenoic acid, *Mortierella alpina*, *Mortierella elongata*, mycelium aging.

Arachidonic acid and eicosapentaenoic (EPA) are important intermediates in eicosanoid metabolism. They are precursors of prostaglandins, leukotrienes and a large group of C20 and C22 compounds that are presently the subject of intrinsic nutritional and medical research (1–4). Supply of EPA from the current major commercial source, fish and fish oils, is unlikely to meet future requirements and alternative microbial sources are being sought (5). Arachidonic acid is presently isolated in low yields from animal adrenal gland and liver as well as from sardines (6). Lower fungi of the Phycomycetes class are a promising source of a variety of polyunsaturated fatty acids (7). These fungi, especially in the order Mucorales, are rich in gamma-linolenic acid, and the fermentation process for production of this fatty acid product has been commercialized (8–10). Many species of the genus *Mortierella* are rich in arachidonic acid and EPA, depending on the species and culture conditions (11–15). We have previously investigated factors affecting production of arachidonic acid and EPA by *Mortierella* strains producing high amounts of these acid components (16,17).

As microbial biomass ages, many microorganisms tend to store their energy source in the form of lipid, and this lipid is usually rich in saturated and monounsaturated fatty acids. With many microorganisms, a general decrease of unsaturated fatty acid as a function of time has been noted (18). However, in the case of a photosynthetic protist *Ochromonas danica* (19), a marine diatom, *Phaeodactylum tricornutum* (20), and some *Mortierella* species (12,15,17), the concentrations of polyunsaturated fatty acids increased significantly as the culture aged. However, factors that affect production of polyunsaturated fatty acids during aging of these species have not been examined. In this paper, we report aspects of this aging phenomenon with respect to production of arachidonic acid by *M. alpina* ATCC 32222 and EPA by *M. elongata* NRRL 5513.

MATERIALS AND METHODS

Chemicals. Standard fatty acids were purchased from Sigma Chemical Company, St. Louis, MO. Solvents and reagents were obtained from Aldrich Chemical Co., Milwaukee, WI, and British Drug House, Toronto, Canada. Linseed oil was supplied by Recochem Inc., Toronto, Canada. It contained (%, w/w): palmitic acid, 4.9; stearic acid, 3.0; oleic acid, 19.5; linoleic acid, 14.5; α-linolenic acid, 53.5; and other acids, 3.7.

Culture conditions. Mortierella strains were maintained on 3% agar containing 20 g/L glucose and 10 g/L yeast extract and were transferred every 2 mon. Culture medium for production of arachidonic acid by *M. alpina* contained (g/L): glucose, 50; yeast extract, 5; sodium nitrate, 3; K_2HPO_4, 1; $MgSO_4 \cdot 7H_2O$, 0.5; KCl, 0.5 and $FeSO_4 \cdot 7H_2O$, 0.01. Medium for production of EPA by *M. elongata* NRRL 5513 contained (g/L): linseed oil, 20; KH_2PO_4, 2.4; KNO_3, 1; $CaCl_2 \cdot 2H_2O$, 0.1; $MgSO_4 \cdot 7H_2O$, 0.5; $FeCl_3 \cdot 6H_2O$, 0.015; $ZnSO_4 \cdot 7H_2O$, 0.0075; and $CuSO_4 \cdot 5H_2O$, 0.0005. Liquid cultures containing 50 mL of these media in 250-mL Erlenmeyer flasks were inoculated with mycelium from freshly grown agar cultures and incubated at 25°C with orbital shaking at 300 rpm. A 5% inoculum from these flasks was used to inoculate similar replicate Erlenmeyer flasks for production of arachidonic acid and EPA. *M. alpina* and *M. elongata* production cultures were incubated at 25°C and 15°C, respectively.

Mycelium aging. Mycelium was harvested by vacuum filtration, and approximately 5 g (wet weight) was stored in small petri dishes at different temperatures for aging.

Biomass determinations. Dry weight of biomass was determined by vacuum filtration or centrifugation of fungal cell suspension, washing it with 1% NaCl and distilled water and drying at 100°C for 12–16 hr.

Extraction and determination of lipids. The dried cells were weighed (20–40 mg) in Teflon-lined screw-cap test tubes of 10 mL capacity, and the lipids were extracted according to the procedure of Bligh and Dyer (21). The extracted lipids were dried at 36°C under nitrogen atmosphere and then methylated by the method of Holub and Skeaff (22). Then the fatty acid methyl esters were dissolved in 200 μL n-hexane, and a 1-μL sample was in-

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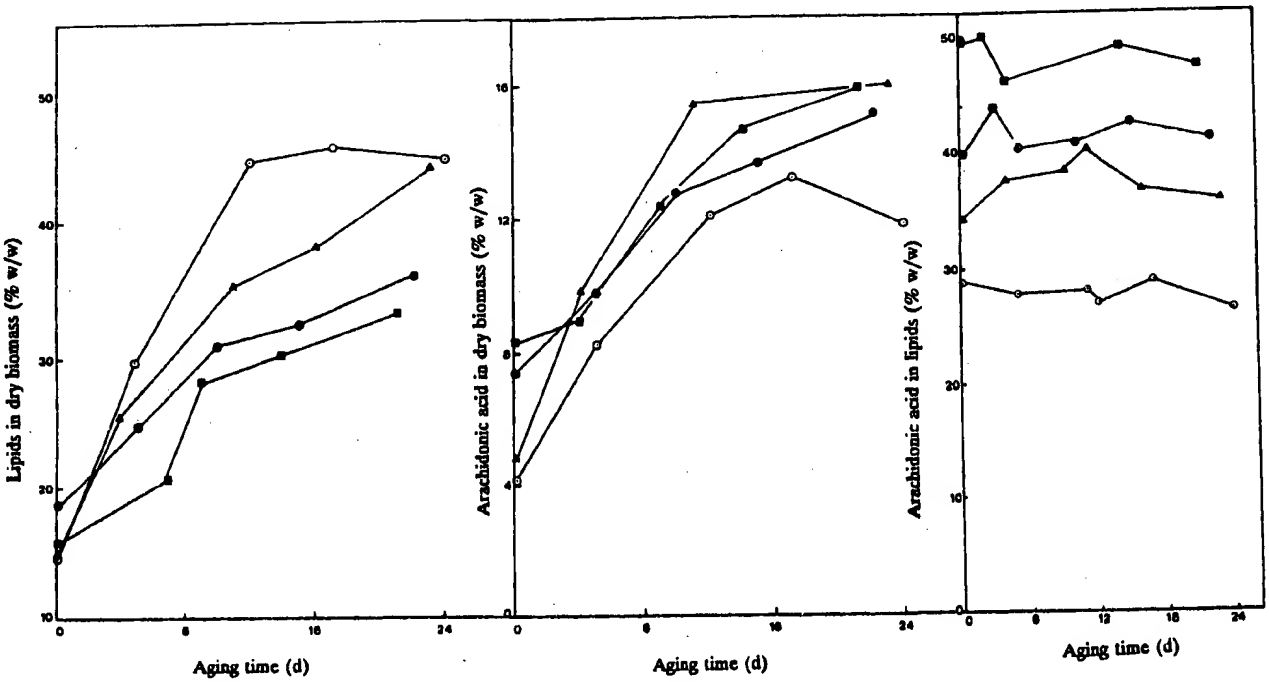

FIG. 1. Content of lipids and arachidonic acid in *M. alpina* biomass as a function of aging time for mycelium harvested after different periods of cultivation. Period of cultivation: ○, 3 days; ▲, 4 days; ●, 5 days; ■, 6 days. Cultivation time: 25°C; aging temperature: 25°C.

jected into a gas-liquid chromatograph (GLC) for analysis. The Shimadzu CR601 GLC (Kyoto, Japan) was connected with a GC-14A data integrator. The GLC was fitted with megabore column DB-225 (Chromatographic Specialties, Brockville, Ontario, Canada) and a flame ionization detector. Helium was used as the carrier gas. The fatty acid ester peaks were identified and calibrated by using standard fatty acids. Pentadecanoic acid (C15:0) was used as internal standard. Analytical data are the averages of three determinations.

RESULTS

Mycelium was harvested from *M. alpina* and *M. elongata* cultures at different times. In previous work (16,17). we have observed that *M. alpina* cultures reached a stationary phase of growth after 3 days at 25°C and that little variation in biomass was observed in these cultures between 3–6 days. Preliminary studies indicated that maximum EPA was produced by *M. elongata* at 15°C in linseed oil-containing media, and that maximum biomass was observed after approximately 7 days. Cultures of *M. alpina* used as a source of arachidonic acid and *M. elongata* as a source of EPA were harvested at intervals from 3–6 days and 7–10 days, respectively, and the effect of aging on the content of these acids was investigated.

Changes in lipid and arachidonic content of *M. alpina* mycelia during aging at 25°C are illustrated in Figure 1. Regardless of culture harvesting time, lipid content in biomass (dry weight basis) increased from 14–18% to 33–45%. Increase in lipid content of mycelium was greatest in cultures harvested after 3 days, and least in 6-day harvested cultures. Initial arachidonic acid content, as a percentage of dry biomass, ranged from 4.1–8.3% (w/w). General increases in arachidonic acid content were observed during the 24-day storage period at 25°C. Maximum arachidonic acid contents in biomass (dry basis) due to aging ranged from 13–16% (w/w). Plots of arachidonic acid content in lipids indicate that during storage the proportion of arachidonic acid in lipids remains relatively constant, ranging from 26–29% in 3-day harvested mycelium to 46–50% in 6-day harvested mycelium.

When the maximum content of lipid and arachidonic acid observed during storage is related to harvesting time, an interesting trend is observed (Fig. 2, Table 1). Maximum lipid content produced in biomass during storage declines as harvesting time is increased from 3–6 days while maximum arachidonic acid content in lipid increases. Maximum arachidonic acid content produced in biomass during storage was found to be relatively independent of harvesting time. The fatty acid spectra corresponding to the 3- and 6-day harvested and stored samples indicate that the 3-day material contains a greater percentage in lipid of palmitic (16:0), stearic (18:0) and oleic (18:1) acids and a lower percentage of arachidonic acid than the 6-day material.

By adjusting the culture pH in the range 4.0–9.0 prior to mycelia harvesting, the effect of pH on lipid and arachidonic acid development during a 6-day storage period at 25°C was investigated (Fig. 3, Table 2). Lipid and arachidonic acid content of biomass was maximum when mycelium was harvested from medium at pH 8, whereas the arachidonic acid content of lipids was highest in mycelia recovered from culture medium adjusted to pH 6. Fatty acid spectra of mycelia stored at pH 4,6 and 8 illustrate that the increased arachidonic acid content of

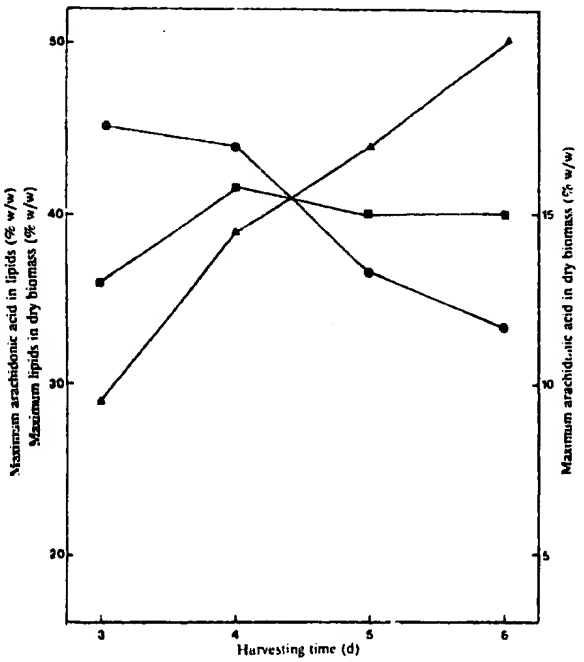

FIG. 2. Effect of culture harvesting time on maximum lipid and arachidonic acid content of *M. alpina* mycelium observed during aging: ▲, arachidonic acid in lipids; ■, arachidonic acid in biomass; ●, lipids in biomass. Cultivation temperature: 25°C; aging temperature: 25°C.

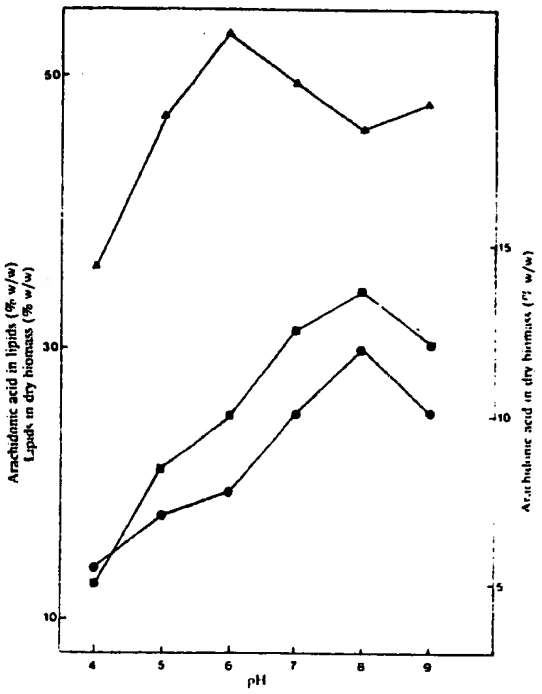

FIG. 3. Effect of adjusting culture pH prior to culture harvesting of lipid and arachidonic acid content of aged *M. alpina* mycelium: ▲, arachidonic acid in lipids; ■, arachidonic acid in biomass; ●, lipids in biomass. Cultivation temperature: 25°C; aging temperature: 25°C; aging time: 15 days.

TABLE 1

Fatty Acid Spectra (% of total fatty acids) of 3- and 6-day Harvested and Stored Samples

	16:0	16:1	18:0	18:1	18:2	18:3	20:3	20:4	20:5	Others
3 Days	12.2	0.2	17.5	14.3	6.1	0.7	5.9	28.9	0.0	14.1
6 Days	8.8	trace	10.2	10.5	4.7	trace	2.7	48.9	0.0	14.1

TABLE 2

Fatty Acid Spectra (% of total fatty acids) of Samples Stored at pH 4, 6 and 8

	16:0	16:1	18:0	18:1	18:2	18:3	20:3	20:4	20:5	Others
pH 4	13.0	trace	14.0	14.6	5.3	trace	2.1	36.0	0.0	15.0
pH 6	8.5	0.8	9.0	9.9	4.9	trace	2.4	51.3	0.0	13.1
pH 8	9.1	trace	10.5	11.3	5.1	trace	2.9	46.2	0.0	15.0

lipid occurs at pH 6 and 8 primarily at the expense of saturated palmitic, stearic and oleic acids. The effect of temperature on aging was investigated at 5, 15 and 25°C (Fig. 4). Overall, temperature appeared to have little effect on total lipid and arachidonic acid content.

The effect of aging time on lipid and EPA content was monitored in mycelia stored at 4°C over a 31-day period (Fig. 5, Table 3). Lipid content in mycelium harvested after 9 and 10 days increased to a maximum after 15–16 days of aging and then manifested a decline. Mycelium harvested after 7 days exhibited a dramatic increase in lipid content between 13 and 31 days of aging. EPA content of biomass and of total cell lipids also increased during aging. The fatty acid spectra of 9-day harvested mycelia before and after storage for 22 days indicate that EPA content in lipids increased 1.73-fold during storage, from 5.1% to 8.8% of total lipids.

The influence of harvesting time on the maximum content of lipids and EPA produced on subsequent aging is illustrated in Figure 6. Maximum lipid content decreased as the harvesting time was extended from 7 to 9 days. Only a small variation in maximum EPA content of aged mycelium as a function of harvesting time was observed. Maximum EPA content in lipid ranged from 7.0–8.4% (w/w) while maximum EPA content in biomass (dry basis) ranged from 28.8–32 mg/g. A limited investigation on the effect of temperature on EPA production during aging was carried out by incubation of the harvested mycelium at 4°C and 15°C (Table 4). Aging temperature manifested little overall effect on EPA contents in lipid and biomass. EPA-containing mycelium was also aged at 25°C. In this case there was essentially no increase in EPA during storage. The effect of adjusting the culture pH prior to harvesting on production of EPA during aging is illustrated in Table 5. The pH curves obtained are relatively flat over the range pH 4–9, with optima for EPA in biomass and in lipids observed at 6 and 7, respectively.

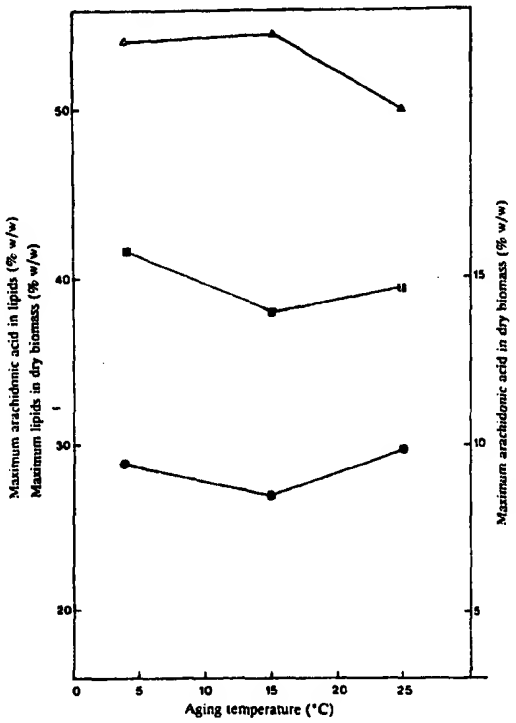

FIG. 4. Effect of storage temperature on maximum yields of lipids and arachidonic acid content observed in *M. alpina* mycelium during aging: ▲, arachidonic acid in lipids; ■, arachidonic acid in biomass; ●, lipids in biomass.

DISCUSSI N

Extending the harvesting time prior to storage to increase the arachidonic acid or EPA content of lipid may facilitate recovery of these acids during downstream processing. Harvesting time had little effect on maximum arachidonic acid or EPA content of aged mycelium. Fatty acid spectra for *M. alpina* harvested after 3 and 6 days indicated a general increase in unsaturation level with increased culture time, and spectra presented for *M. elongata* before and after aging also indicated a shift to more polyunsaturated fatty acids during storage. Aging of harvested *M. alpina* for 6 days resulted in decomposition of the major mycelial fatty acids, palmitic, oleic and linoleic acid with arachidonic acid content of lipids concomitantly rising to nearly 70% of total fatty acids (12). Nevertheless, there is a general lack of data in the literature on changes in mycelial lipid composition following harvesting, with most reports describing effects of environmental conditions on fungal lipids during culture. Our results will, therefore, be discussed in the context of these reports.

The amount of lipid produced by a given species of fungus has been found to depend to a great extent on the developmental stage of growth and/or culture conditions (23). In the case of *A. nidulans*, fat formation in surface cultures at 25°C was found to accelerate at the later stages of growth after which fat content decreased (24). Boulton and Ratledge (25) described a general biphasic pattern of lipid accumulation in oleaginous organisms during batch culture. When nutrients are in excess, lipid content of cells stays approximately constant, while after nutrients and

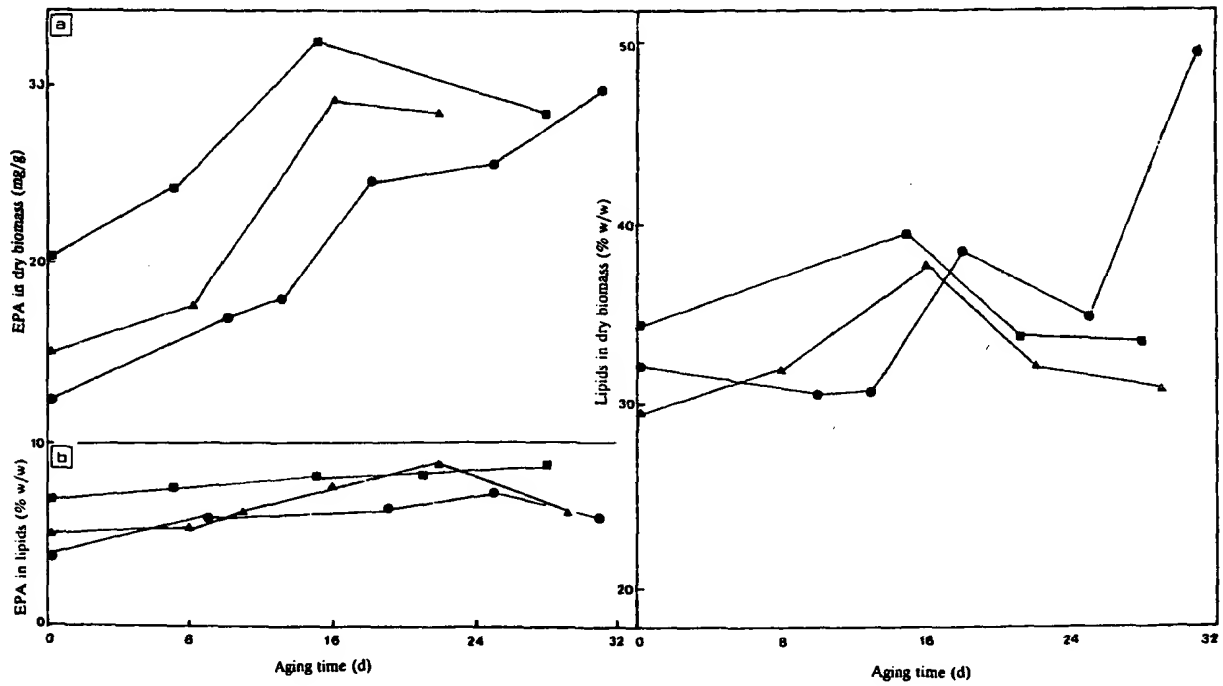

FIG. 5. Content of lipids and EPA as a function of aging time in *M. elongata* mycelium harvested after different periods of cultivation. Period of cultivation: ●, 7 days; ▲, 9 days; ■, 10 days. Cultivation temperature: 15°C; aging temperature: 4°C.

TABLE 3

Fatty Acid Spectra (% of total fatty acids) of 9-day Harvested Mycelia Before and After Storage for 22 days

	16:0	16:1	18:0	18:1	18:2	18:3	20:4	20:5	Others
Before	7.0	trace	3.3	21.8	13.6	34.8	5.3	5.1	9.2
After	6.6	0.4	2.6	18.2	12.3	30.7	8.3	8.8	12.2

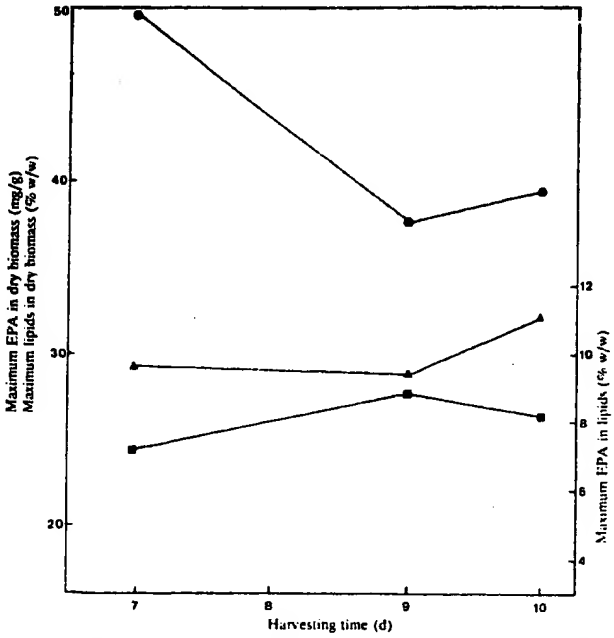

FIG. 6. Effect of culture harvesting time on maximum lipid and EPA content of *M. elongata* mycelium observed during aging: ▲, EPA in lipids; ■, EPA in biomass; ●, lipids in biomass. Cultivation temperature: 15°C; aging temperature, 4°C.

especially nitrogen are exhausted there is a continued build-up of lipid without a corresponding increase in biomass.

Maximum lipid and arachidonic acid was produced in *M. alpina* mycelia aged at pH 8, while maximum lipid and EPA was observed in *M. elongata* mycelium stored at pH 7 and 6, respectively. An increase in unsaturated fatty acids with increasing culture pH has been reported in many fungi (26).

It was noted that the temperature of aging had little effect on lipid, arachidonic acid (in the range 5–25°C) and EPA (in the range 4–15°C) contents of *M. alpina* and *M. elongata*. No increase in EPA content of *M. elongata* was observed during aging at 25°C. In cell culture studies, generally lower temperatures result in an increase in unsaturation (27,28). Lipids of *Mucor* and *Rhizopus* species were more unsaturated when fungi were grown at lower temperature (23).

Harvesting of mycelium from liquid culture media may have the effect of increasing oxygen supply to the stored mycelium since increased oxygen tension elevated unsaturated fatty acid content of fungi of the order Mucorales (29). The desaturase enzymes required for production of the unsaturated fatty acids require molecular oxygen as cofactor (27). The tendency for some fungi to produce polyunsaturated fatty acids at low temperatures may be due to the temperature-labile nature of desaturase enzymes (30). This may explain the observation that aging of *M. elongata* mycelium at 25°C did not increase EPA content.

The aging studies reported in this paper may have a practical application in the development of fungal systems for commercial production of arachidonic acid, EPA, and possibly DHA.

TABLE 4

Effect of Storage Temperature on Lipids and EPA Content Observed in *M. elongata* Mycelium During Aging[a]

Storage temperature (°C)	Component	Aging time (days) 0	7	8	15	21	28
4	EPA in dry biomass	24.0	20.2	—	32.0	28.0	28.0
15	(mg/g)	24.0	20.2	—	27.2	33.0	30.2
4	EPA in lipids	7.0	7.6	—	8.1	8.3	8.4
15	(% w/w)	7.0	6.7	—	7.4	—	8.1
4	Lipids in dry biomass	34.4	—	26.6	39.2	33.6	33.6
15	(% w/w)	34.4	—	30.0	36.6	33.0	30.2

[a]Cultivation time: 10 days; cultivation temperature: 15°C.

TABLE 5

Effect of Adjusting Culture pH Prior to Culture Harvesting on Lipid and EPA Content of Aged *M. elongata* Mycelium[a]

Component	Adjusted pH 4	5	6	7	8	9
EPA in dry biomass (mg/g)	26.6	31.2	34.8	31.6	30.4	28.2
EPA in lipids (% w/w)	8.8	10.0	9.0	10.9	9.6	8.1
Lipids in dry biomass (% w/w)	31.2	31.2	38.5	29.4	31.8	34.8

[a]Cultivation time: 10 days; storage time: 6 days; cultivation temperature: 15°C; storage temperature: 4°C.

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Production of the polyunsaturated fatty acids arachidonic acid and eicosapentaenoic acid by the fungus *Pythium ultimum*

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Several strains of species of the fungal genus *Pythium*, and of *Phytophthora cinnamomi*, were screened for content of the polyunsaturated fatty acids (PUFAs) arachidonic acid (AA) and eicosapentaenoic acid (EPA). The aim of the investigation was to establish alternative sources of these PUFAs, which are of importance in human nutrition. As a relatively prolific producer of EPA and AA, *P. ultimum* strain #144 was selected for a study of conditions that enhance their production over baseline levels that are present in the fungus when cultured for 6 d at 25 °C with rotary shaking (120 r.p.m.) in Vogel's medium containing sucrose as the carbon substrate. The levels of AA and EPA under these conditions were 133 ± 27 and 138 ± 25 mg l^{-1} ($n = 5$), respectively. Maximal production of these fatty acids was accomplished by the following sequence of steps. (1) Incubate the cultures for 6 d after inoculation under the conditions described above. Then (2) add glucose to the cultures (2%, w/v, final concentration) and incubate for a further 6 d at 13 °C. Under these conditions, the AA content of the mycelium was 205% higher than baseline levels and the EPA content was 198% higher. (3) Allow the cultures to remain stationary for 10 d which increases the AA content to 253% above baseline levels and the EPA content by 236%. Using such a procedure, 322 mg AA l^{-1} and 383 mg EPA l^{-1} were produced.

Introduction

Beneficial health effects of consuming certain marine fish and fish oils are attributed to the presence of the ω-3 fatty acids 5,8,11,14,17-eicosapentaenoic acid (EPA) and 4,7,10,13,16,19-docosahexaenoic acid (DHA) (Sanders, 1988; Simopoulos, 1989). ω-3 fatty acids exhibit beneficial effects in coronary heart disease, hypertension, inflammation, arthritis, psoriasis, other autoimmune disorders and cancer (Simopoulos, 1989). At present, the oils of certain marine fish are the only commercial sources of EPA and DHA. This has prompted a search for other sources of such polyunsaturated fatty acids (PUFAs) (Yongmanitchai & Ward, 1989).

Possible alternate sources of the ω-3 fatty acids include certain algae (Erwin, 1973; Behrens *et al.*, 1989; Yongmanitchai & Ward, 1989), which are actually the dietary origin of such fatty acids in fish. Although restricted in occurrence, EPA appears to be more widespread in nature than DHA. It has been reported in certain marine bacteria (Yazawa *et al.*, 1988) and some of the more primitive fungi such as the Oomycetes, including the pythiaceous species (*Pythium* and *Phytophthora*) (Shaw, 1966), and the Zygomycete genus *Mortierella* (Yamada *et al.*, 1987*a*, *b*).

Arachidonic acid (AA) is a biogenic precursor to a wide variety of biologically active eicosanoids such as prostaglandins and thromboxanes (Nelson *et al.*, 1982). This fatty acid is usually obtained from pig liver and adrenal glands (Ratledge, 1989). It appears to be more widely distributed than EPA and DHA, having been identified as a product of animals and numerous microorganisms such as certain protozoa, amoebae, algae and fungi. With few exceptions, AA distribution in fungi appears to be mainly in the more primitive species such as those belonging to the classes Hyphochytriomycetes, Chytridiomycetes and Oomycetes of the subdivision Mastigomycotina (Weete *et al.*, 1989), including *Pythium ultimum* (Kerwin & Duddles, 1989). It has also been found in *Mortierella* spp. (Yamada *et al.*, 1987*a*, *b*; Shinmen *et al.*, 1989). Low levels of AA have been reported (Radwan & Soliman, 1988) in species of some of the more advanced fungal genera, i.e. *Penicillium*,

Abbreviations: AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FAME, fatty acid methyl ester; LO, linseed oil; PUFA, polyunsaturated fatty acid.

Aspergillus and *Fusarium*, when cultured on short-chain fatty acids.

Results of preliminary experiments in this laboratory indicated that certain *Pythium* species may have potential for the production of relatively high amounts of EPA and AA. The objective of the research reported here was to find a high EPA- and/or AA-producing strain(s) or species of *Pythium*, and to develop laboratory culture procedures for maximizing the production of these fatty acids.

Methods

Sources of fungi and culture conditions. *Pythium aphanidermatum* and *Pythium myriotylum* were obtained from M. C. Rush, Department of Plant Pathology and Crop Physiology, LSU, Baton Rouge, LA, USA; a complex of *Pythium irregulare* and *Pythium pareocandrum*, and *Pythium sylvaticum* were obtained from D. J. S. Barr (see Table 1). Otherwise, the sources of *Pythium ultimum* and *Phytophthora cinnamomi* are given in Table 1.

Mycelia of each strain were stored at −70 °C in Vogel's medium containing 10% (v/v) glycerol. Unless noted otherwise, they were cultured for 6 d at 25 °C in 250 ml Erlenmeyer flasks containing 100 ml Vogel's defined medium (Vogel, 1964) which has sucrose and ammonium nitrate as carbon and nitrogen sources, respectively. Cultures were agitated on a New Brunswick or Lab-line rotary incubator shaker at 120 r.p.m. Inoculations were made with 5 ml of blended mycelium from a 6-d-old culture grown as described above. Mycelia were harvested by suction filtration in a Buchner funnel, washed twice with 100 ml cold 0·1 M-potassium phosphate buffer (pH 7·0) each, and dried by lyophilization prior to extraction. In experiments where the fungus was grown at different temperatures beginning immediately after inoculation, the cultures used for inoculum were adapted to the requisite temperatures by two successive transfers and subsequent incubation for 6 d after each.

The effects of other nutrients on growth and lipid production was determined by growing the mycelia in Vogel's medium in which the sucrose was replaced by either 2% (w/v) glucose, 2% (w/v) maltose, or corn steep liquor (1% or 2%, v/v). The fungus was also cultured in Vogel's medium supplemented with 1% (v/v) bovine milk whey, 4% (v/v) V-8 (blended vegetables) juice or 1% (v/v) linseed oil. Also, potassium nitrate, urea or glycine were substituted for ammonium nitrate at equivalent nitrogen contents. In addition, mycelia were incubated in media with high carbon/nitrogen ratios or cultured in 2% (w/v) potato dextrose, 2% (w/v) malt dextrose and 1% yeast extract/2% dextrose media. With the exception of glucose in combination with low temperature (see below), none of these media components or treatments had an appreciable effect on total lipid content, or the AA and EPA content of the lipid, and therefore the data are not given.

To study the effects of glucose and temperature on EPA and AA production, mycelia were first grown in Vogel's medium for 6 d at 25 °C, and then sterile glucose solution was added to the culture medium to a final concentration of 2%. The mycelia was then incubated for an additional 6 d at either 25 °C or 13 °C before harvesting. The controls received equal volumes of sterile distilled water in place of the glucose solution.

For the evaluation of data to determine conditions favouring AA and/or EPA production, values for growth, lipid and fatty acid contents were compared to 'baseline' data from fungi cultured in Vogel's medium under the conditions described above. Each experiment was done at least twice, and the data given in this paper are representative. The values given for each experiment are means of two to three treatment replications. Overall baseline values for AA and EPA content of *P. ultimum* strain #144 are means of five separate experiments, and the standard deviations reflect variations among these experiments.

Extraction and analytical procedures. Lipid was extracted from mycelium (ca. 50 mg) using the procedure of Bligh & Dyer (1959). In the case of dry tissue, the mycelium was hydrated with 1·0 ml water prior to extraction. The amount of lipid extracted was measured gravimetrically.

A portion (ca. 5 mg) of the total lipid was methylated by heating the sample with 0·5 M-sodium methoxide in dry methanol for 20 min at 75 °C according to the procedure provided by Applied Science Laboratories (Deerfield, IL, USA). The fatty acid methyl esters (FAMEs) were analysed by gas-liquid chromatography as described previously (Weete *et al.*, 1989) except that the gas chromatograph was equipped with a 30 m × 0·25 mm fused silica capillary column (J. & W. Scientific) coated with DB-225 (50% cyanopropylmethyl, 50% methyl phenyl silicone). The injector and detector temperatures were 250 °C, and the oven temperature was programmed from 180 °C to 220 °C at 1 °C min^{-1}. FAMEs were identified by comparison of their retention times relative to methyl heptadecanoate with those of authentic standards obtained from Applied Science Laboratories, and they were quantified by the internal standard method using the same standard. The identities of AA and EPA have been confirmed by mass spectrometry (data not shown).

Results and Discussion

Screening

Twenty-three strains of *Pythium ultimum*, seven strains of *Pythium aphanidermatum*, three other species of *Pythium* and *Phytophthora cinnamomi* were screened for growth, total lipid production, and their AA and EPA contents. Since values for the other species and strains (see Methods) were generally lower, only the results for *P. ultimum* are given. Total lipid production varied among strains from 0·75 g l^{-1} for strain #1786 to 2·01 g l^{-1} for #650 (Table 1). Several strains stood out from the others tested in terms of AA and EPA production, e.g. #128, #144, #418, #511, #583, #639, #640 and #650 at 110–230 mg AA l^{-1} and 150–220 mg EPA l^{-1} (Table 1). The AA/EPA ratios ranged from 0·44 to 3·00 (Table 1). The lipid from these strains contained more EPA than does Menhaden oil, e.g. lipid from strain #144 contained 70% more EPA than this fish oil. Although *Ph. cinnamomi* contained relatively high amounts of AA and EPA, the growth rate was slower than that of some of the most productive *Pythium* species, and therefore *Ph. cinnamomi* was not studied further.

Although Shaw (1966) suggested that the pythiaceous fungi are capable of producing the long-chain PUFAs AA and EPA, Bowman & Mumma (1967) were unable to detect these fatty acids in *P. ultimum*. They reported finding relatively high amounts of $C_{22:1}$ and $C_{22:2}$. More

Table 1. *Total lipid, arachidonic acid (AA) and eicosapentaenoic acid (EPA) contents of Pythium species and strains, and of Phytophthora cinnamomi*

All of the cultures were grown in Vogel's medium for 6 d at 25 °C with rotary shaking at 120 r.p.m., except for *Ph. cinnamomi* which was grown for 12 d under similar conditions. Values for *P. ultimum* strains #128 to #650 (denoted *a*) are means of two experiments and three replicates each. All the remaining values are means of three replicates of one experiment. The variation among replicates and experiment was less than 10%.

Species and strain #*	Total lipid ($g\ l^{-1}$)	AA ($mg\ l^{-1}$)	EPA ($mg\ l^{-1}$)	AA/EPA ratio
Pythium ultimum				
128[a]	1·46	140	150	0·93
144[a]	1·94	230	160	1·44
319[a]	1·49	70	100	0·70
406[a]	1·68	120	90	1·33
418[a]	1·78	110	210	0·52
425[a]	1·45	80	130	0·62
433[a]	1·90	140	150	0·93
447[a]	1·27	100	150	0·67
471[a]	1·25	90	80	1·13
511[a]	1·90	190	210	0·91
583[a]	1·33	140	140	1·00
600[a]	1·13	40	40	1·00
612[a]	1·44	100	100	1·00
634[a]	1·60	90	100	0·90
638[a]	1·68	100	90	1·11
639[a]	1·55	150	190	0·79
640[a]	1·83	170	220	0·77
1520[b]	1·33	40	90	0·44
23–5[b]	1·33	50	70	0·71
20–2[b]	0·97	60	40	1·50
1417[b]	0·81	50	40	1·25
1696[c]	1·00	110	50	2·20
1786[c]	0·75	90	30	3·00
650[a] (var. *sporangiferum*)	2·01	140	90	1·56
var. *ultimum*[d]	0·92	80	100	0·80
var. *sporogonium*[d]	0·52	20	20	1·00
Pytophthora cinnamomi[e]	1·12	110	140	0·79

* Sources of strains were as follows: *a*, D. J. S. Barr, Biosystematic Research Center, Central Experimental Farm, Ottawa, Canada; *b*, M. C. Rush, Dept. of Plant Pathology and Crop Physiology, Baton Rouge, LA, USA; *c*, E. Butler, Dept. Plant Pathology, University of California at Davis, CA, USA; *d*, A. J. Latham, Dept. of Plant Pathology, Auburn Univ., AL, USA; *e*, E. A. Curl, Dept. of Plant Pathology, Auburn Univ., AL, USA.

recently, however, Kerwin & Duddles (1989) reported both AA and EPA in this species.

Lipid and fatty acid contents as a function of culture age, temperature and shaker rate

Based on the results presented above, *P. ultimum* strain #144 was selected for further study. When incubated at 13, 18 and 25 °C in Vogel's medium, the cultures reached the stationary phase 4–6 d after inoculation. The inocula for these cultures had been pre-adapted at the respective temperatures. Although maximum biomass production at the different temperatures occurred in the order 18 °C > 13 °C ≥ 25 °C, the biomass per flask at 18 °C was only about 20% more than that at 25 °C (Table 2), and about 30% higher than that at 30 °C (data not shown). The lipid content increased rapidly during growth and did not change appreciably during the stationary phase, i.e. for a total of 10 d incubation. Cultivation at 18 °C or above favoured lipid accumulation compared to that at 13 °C, but the highest lipid content of 26% (on a dry weight basis) occurred after 6 d growth at 25 °C (Table 2).

Table 2. *Production of total lipid, arachidonic acid (AA) and eicosapentaenoic acid (EPA) by P. ultimum strain #144 as a function of temperature*

Cultures were incubated for 6 d at the respective temperatures. Values are means of two experiments with three replicates each. The variation among experiments and replicates was less than 8·0%.

Temperature (°C)	Biomass ($g\ l^{-1}$)	Total lipid ($g\ l^{-1}$)	AA ($mg\ l^{-1}$)	EPA ($mg\ l^{-1}$)
13	8·3	1·0	29	100
18	8·9	2·2	133	170
25	7·4	2·0	220	170

The fatty acid composition changed with growth temperature (Table 3). The most notable differences were in the $C_{18:1}$ content, which was 20% at 25 °C compared to 33% at 13 °C, and the AA content which decreased from 14·6% to 3·3% with the same temperature change (Table 3). The $\Delta\ mol^{-1}$ value, an indication of the degree of unsaturation (see Table 3), of 1·6 was the same for total fatty acids from mycelium cultured at 13 °C and 18 °C, in spite of a higher $C_{18:2}$ content and correspondingly less $C_{18:1}$, and a higher $C_{18:3}$ and a lower $C_{20:4}$ content at 18 °C compared to 13 °C (Table 3). The $\Delta\ mol^{-1}$ value was 1·9 at 25 °C which was due mainly to the relatively high $C_{20:4}$ content. Also, $C_{20:1}$ was detected in cultures grown at the two higher temperatures, but not at 13 °C. Although the AA content increased progressively with increasing temperature, the relative proportion of EPA was similar at the three temperatures tested. However, although EPA production was essentially the same at about 170 $mg\ l^{-1}$ at 18 °C and 25 °C, it was 42% less at 13 °C (Table 2). AA production was progressively less by 87% from 25 °C to 13 °C. Because of the differential response of AA and EPA to temperature, the ratio of these two fatty acids in the oil may be regulated by temperature.

Table 3. *Total fatty acid composition (weight%) of Pythium ultimum strain # 144 as a function of cultivation temperature*

Values are means of two experiments with three replicates each. The variation among experiments and replicates was less than 5%.

Fatty acid	Temperature (°C)		
	13	18	25
$C_{14:0}$	9·3	9·1	7·0
$C_{14:1}\Delta^{9}$	–	0·3	0·3
$C_{16:0}$	12·0	15·8	15·4
$C_{16:1}\Delta^{9}$	6·4	6·3	4·8
$C_{18:0}$	2·0	1·4	2·0
$C_{18:1}\Delta^{9}$	33·0	22·8	20·1
$C_{18:2}\Delta^{9,12}$	13·2	17·2	15·9
$C_{18:3}\Delta^{6,9,12}$	6·5	1·4	1·1
$C_{18:3}\Delta^{9,12,15}$	0·8	0·9	0·7
$C_{20:0}$	–	0·4	0·6
$C_{20:1}\Delta^{11}$	–	5·5	4·3
$C_{20:2}\Delta^{11,14}$	–	0·2	0·5
$C_{20:3}\Delta^{8,11,14}$	1·3	0·6	0·9
$C_{20:4}\Delta^{5,8,11,14}$	3·3	7·5	14·6
$C_{20:5}\Delta^{5,8,11,14,17}$	11·0	11·0	11·5
$C_{22:1}\Delta^{13}$	0·0	0·1	0·1
Δ mol^{-1}*	1·6	1·6	1·9

* Calculated using the equation Δ mol^{-1} = 1(monoenes) + 2(dienes) + 3(trienes) + 4(tetraenes) + 5(pentaenes)/100.

Cultivation time (culture age) had little effect on the relative proportion of most fatty acids during 6 d growth at 25 °C. The noteworthy changes in fatty acid composition with time were a decrease in $C_{18:1}$ and increases in AA and EPA after 2 d incubation, but these changes had little effect on the Δ mol^{-1} value which was similar throughout the growth period at 1·7 to 1·8. Increasing the shaker rate from 120 to 300 r.p.m., at either 13 °C or 25 °C, beginning at inoculation, either had no effect or only a slight beneficial effect on biomass, total lipid, AA or EPA production (data not given).

When cultured under baseline (see Methods) conditions, *P. ultimum* strain #144 produced 133 ± 27 mg AA l^{-1} and 138 ± 25 mg EPA l^{-1} ($n = 5$). Both AA and EPA tended to be higher in the polar lipid fraction than in the neutral lipid fraction as a percentage of total fatty acids, but most of these PUFAs were found in the neutral lipid fraction because the neutral/polar lipid ratio was about 84:16 (data not shown).

Enhancement of AA and EPA production

With the exception of certain species and strains of the zygomycetous genus *Mortierella*, fungi have not been considered previously as potential sources of AA or EPA. Under conditions considered optimum for growth (20–28 °C), the *Mortierella* species studied produce no detectable EPA, but EPA production was induced by cultivation at lower temperatures. Using a relatively high initial growth temperature for biomass production, and intermittent glucose feeding followed by a temperature shift to 12 °C, EPA production by strains of *M. alpina* was brought to 490 mg l^{-1} (Yamada *et al.* 1987*b*; Shimizu *et al.*, 1988*a*). Certain species and strains of *Mortierella* are also capable of producing relatively large quantities of AA. For example, *M. elongata* 1S-4 cultured in 2% glucose/0·5% yeast extract medium produced 3·6 g AA l^{-1} [80 mg (g^{-1} dry wt)$^{-1}$] (Yamada *et al.*, 1987*b*), and strain 1S-5 cultured on 10% glucose/0·5% polypeptone/0·3% yeast extract medium produced 0·99 g AA l^{-1} (Yamada *et al.*, 1987*a*). Unlike EPA production, AA production in *Mortierella* did not require a low temperature. The relative proportion of AA was increased from 31% of the total fatty acids to 70% by allowing the harvested mycelia to stand for 6 d (Shinmen *et al.*, 1989).

A series of preliminary experiments were done with the aim of enhancing AA and/or EPA production in *P. ultimum*. Some previous results indicated that although growth and total lipid were lower with glucose in place of sucrose as the carbon source, the relative proportion of EPA in the total fatty acid fraction was moderately increased. Also, although cultures grown at 13 °C beginning at inoculation contained less total lipid, AA and EPA were slightly enriched in the lipid at this temperature. Therefore, biomass was allowed to accumulate in Vogel's medium for 6 d at 25 °C, and then glucose at 2% final concentration was added to the culture medium. This was followed by an additional 6 d incubation that resulted in a 19% and 37% increase, on a mg l^{-1} basis, in AA and EPA production, respectively, over that at 6 d with sucrose only. These values were increased to 99% and 129%, respectively, when glucose was added as before except that the cultures were incubated at 13 °C instead of 25 °C. The cultures had not been previously adapted to the lower temperature. Also, allowing the cultures (Vogel's medium, 25 °C) that had been incubated for 6 d with shaking to remain stationary for an additional 10 d increased AA and EPA production by 55% and 58%, respectively.

In the light of results from the separate experiments described above, experiments were done that incorporated the above conditions in an effort to maximize AA and EPA production. In a representative experiment, baseline values for AA and EPA production were 91 and 114 mg l^{-1}, respectively (Table 4). Extending the incubation time from 6 to 12 d at 25 °C had relatively little effect on lipid and AA or EPA production, i.e. a 32% decrease in AA and a slight increase in EPA. However, AA production and EPA production (mg l^{-1}) were increased by 14% and 68%, respectively, over the baseline values in cultures to which 2% glucose was

Table 4. *Effect of temperature, glucose and agitation conditions on total lipid, arachidonic acid (AA) and eicosapentaenoic acid (EPA) contents of Pythium ultimum*

All cultures were initially incubated in Vogel's medium at 25 °C under shake conditions. At 6 d after inoculation, some cultures received water and some glucose to a final concentraton of 2%. These flasks were incubated for a further 6 d at either 25 °C or 13 °C. After a total of 12 d incubation, some cultures were harvested for analysis and others were incubated under stationary conditions for an additional 10 d prior to harvest. Baseline values were from mycelia cultured in Vogel's medium for 6 d at 25 °C, with rotary shaking at 120 r.p.m. Values are means of three replications of one representative experiment. The variation among replicates was less than 10%.

Temperature of second incubation (°C)	Incubation conditions	Dry wt ($g\ l^{-1}$)	Total lipid ($g\ l^{-1}$)	AA ($mg\ l^{-1}$)	EPA ($mg\ l^{-1}$)
25	Baseline values	7·57	1·1	91	114
	H_2O	7·14	1·2	62	126
	Glucose	9·12	1·6	104	191
	H_2O + 10 d	7·20	1·3	104	166
	Glucose + 10 d	9·13	1·8	214	292
13	H_2O	6·92	1·3	123	175
	Glucose	11·61	2·3	277	340
	H_2O + 10 d	7·09	1·3	136	165
	Glucose + 10 d	11·20	2·0	322	383

added and incubated for an additional 6 d at 25 °C (Table 4). The production of AA and EPA was increased by 35% and 54%, respectively, by transferring baseline cultures from 25 °C to 13 °C for the second 6 d incubation, and was increased by 204% and 198%, respectively, by incubating for the additional 6 d in the presence of glucose at 13 °C. Allowing the 12-d-old cultures without glucose, grown at either 25 °C or 13 °C, to remain stationary for a further 10 d did not result in as much AA or EPA as in the corresponding cultures not allowed the additional stationary incubation period, except in the case of the water controls at 13 °C where there was a slight increase in AA (Table 4). On the other hand, cultures with glucose added and allowed to stand as described above at 25 °C contained 135% and 156% more AA and EPA, respectively, than the baseline cultures. At 13 °C, glucose-supplemented cultures allowed to stand an additional 10 d produced 254% and 236% more AA and EPA, respectively, than the baseline cultures (Table 4).

With this strategy, we were able to stimulate EPA production by *P. ultimum* strain #144 from the baseline level of about 114 $mg\ l^{-1}$ ($138 \pm 25\ mg\ l^{-1}$, $n = 5$) to 340 $mg\ l^{-1}$. In order to reach this level of production, it was necessary to generate biomass using sucrose as the carbon source, and then stimulate additional EPA production by adding glucose and incubating the cultures at a lower temperature. EPA production could be further enhanced to 383 $mg\ l^{-1}$ by allowing glucose-fed, low temperature-treated cultures to remain stationary for 10 d after the 12 d incubation under shake culture conditions. It has been found that glucose in a yeast and malt extract/peptone/salts medium gave the best mycelial and lipid yield in *Pythium irregulare*; the highest EPA yield in this species with cold temperature and glucose treatment was 112 $mg\ l^{-1}$ (E. E. Stinson, R. Vosacek and M. J. Durarity, personal communication). In this case, cultures were incubated at the low temperature beginning at inoculation and the cultures were not allowed to remain stationary prior to harvest. The apparent superiority of *Mortierella* spp. over *Pythium* spp. in EPA production appears to be due mainly to the higher growth capacity of the former rather than the amount of fatty acid produced per unit of biomass. For example, the enhanced level of EPA in *P. ultimum* strain #144 in this study was 22 mg (g dry wt)$^{-1}$ compared to 27–29 mg (g dry wt)$^{-1}$ for *M. alpina* (Shimizu *et al.*, 1988*a*, *b*).

It is well-known that poikilothermic micro-organisms adapt to lower temperature by increasing the degree of fatty acid unsaturation, presumably as a means of modulating membrane fluidity (Thompson, 1985). Therefore, it was unexpected that *P. ultimum* strain #144 did not respond accordingly during the most rapid growth phase when cultured at lower temperatures (<25 °C) beginning just after inoculation, i.e. relative EPA levels were unchanged and the AA content was the opposite of what might be expected at a lower temperature (Table 3). Considering the temperature effect alone, production of AA and EPA was enhanced only when the cultures were shifted to the lower temperature after growth had slowed substantially (stationary phase) which was unexpected in view of the results described above.

The higher AA and EPA contents resulting from this treatment were due mainly to an increase in total lipid. Shimizu *et al.* (1988*b*) attributed the stimulation of EPA production in *Mortierella* spp. to low temperature activation of enzyme(s) involved in EPA synthesis.

Shimizu *et al.* (1989) found that EPA production could be stimulated to 1·35 $g\ l^{-1}$ in *M. alpina* strain 20-17 by supplementing the culture medium with 1% linseed oil (LO). The rationale for this was that LO contains a relatively high amount of α-linolenic acid that would be expected to be converted to EPA. This was also tested with *P. ultimum* strain #144 but, although LO was a good cosubstrate for growth in that biomass production was greater than in Vogel's medium alone, the increase in EPA production was minimal and not proportional to the increase in biomass (data not shown). Since LO was

readily degraded and utilized for growth by *Pythium*, and α-linolenic acid was incorporated into phospholipids and therefore presumably available for elongation and desaturation (data not given), the reason for the inability of LO to promote EPA production in *P. ultimum* strain #144 is unclear.

AA production in *P. ultimum* strain #144 was also stimulated by the same conditions that promote EPA production in this fungus, with a maximum stimulation of 253% over baseline levels. Unlike EPA, which *Mortierella* spp. and *P. ultimum* strain #144 generally produced in similar amounts on a mycelial dry wt basis, AA production by *Pythium* [30 mg (g dry wt)$^{-1}$], at least under the conditions described here, was 62% less than that of *M. elongata* IS-4 (Yamada *et al.*, 1987*b*).

In conclusion, *P. ultimum* strain #144 and certain other strains produce relatively high levels of both AA and EPA under cultivation conditions optimum for growth. Production of these PUFAs can be enhanced substantially by altering the cultivation conditions as described herein. These results suggest that this fungal species (strains) may have potential for commercial development for the production of these fatty acids by fermentation techniques.

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Production of Odd Chain Polyunsaturated Fatty Acids by *Mortierella* Fungi

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A soil isolate, *Mortierella alpina* 1S-4, was found to show high production of odd chain polyunsaturated fatty acids (PUFAs) among various arachidonic acid-producing *Mortierella* strains tested. The fungus mainly accumulated 5,8,11,14-*cis*-nonadecatetraenoic acid. With 5% *n*-heptadecane and 1% yeast extract as growth substrates, the amount of $C_{19:4}$ acid accumulated reached 44.4 mg/g dry mycelia (0.68 mg/mL of culture broth). This value accounted for 11.2% of the total fatty acids in the extracted lipids from mycelia, and odd chain fatty acids comprised over 95% of the total mycelial fatty acids. The addition of sesamin, a specific inhibitor of Δ5 desaturation, caused an increase in $C_{19:3}$ acid and an accompanying decrease in $C_{19:4}$ acid. On the other hand, species of *Mortierella* that could not produce C-20 PUFAs accumulated C-17 acids, but no C-19 PUFAs, when grown with fatty substrates with an odd chain skeleton. The odd chain PUFAs were distributed in both neutral and polar lipids. The biosynthetic route to $C_{19:4}$ acid was presumed to mimic the n-6 route to arachidonic acid as follows: $C_{17:0} \rightarrow C_{17:1} \rightarrow C_{17:2} \rightarrow C_{17:3} \rightarrow C_{19:3} \rightarrow C_{19:4}$ acids.

KEY WORDS: Arachidonic acid, biosynthesis of polyunsaturated fatty acids, *Mortierella alpina*, nonadecatetraenoic acid, odd chain polyunsaturated fatty acids.

The natural occurrence of straight-chain, odd-numbered polyunsaturated fatty acids (PUFAs) has been reported in ruminant, fish and several other animal lipids. They can be considered as normal constituents of such fats. However, the odd chain PUFAs reported accounted for only a small percentage of the total cellular fatty acids. Only mullet oil so far has been reported to be a relatively rich source of these fatty acids, such as C-17 and C-19 PUFAs (1). The fatty acid methyl esters containing mainly 9,12-$C_{17:2}$ and 6,9,12-$C_{17:3}$ acids prepared from mullet oil, as well as linoleic acid methyl ester, were shown to cure the external symptoms of fat-deficient rats and to be converted to 5,8,11,14-$C_{19:4}$ acid in the rat liver (2,3). On the other hand, some mucoralean fungi (4) and *Candida* yeasts (5), grown with odd chain *n*-alkanes, have been shown to accumulate C-17 unsaturated fatty acids such as $C_{17:1}$, $C_{17:2}$ and $C_{17:3}$ acids. However, they do not produce C-19 PUFAs. In our recent studies (6–17), it was found that several fungal strains belonging to the genus *Mortierella* accumulate C-20 PUFAs, such as dihomo-γ-linolenic, arachidonic and eicosapentaenoic acids, in their mycelia. Here we report that these arachidonic acid producers accumulate specifically large amounts of C-17 and C-19 PUFAs in their mycelia when grown with odd chain *n*-alkanes. The identification of the odd chain PUFAs and their distribution in fractionated lipids are also described.

MATERIALS AND METHODS

Chemicals. *n*-Alkanes were purchased from Wako Pure Chemicals (Osaka, Japan). (+)Sesamin was prepared from unroasted sesame oil (S. Shimizu, K. Akimoto, Y. Shinmen, M. Sugano and H. Yamada, unpublished data). All other reagents used in this work were of analytical grade and commercially available.

Microorganisms. All fungal strains used were from our stock cultures (AKU Culture Collection, Faculty of Agriculture, Kyoto University). Each fungus was cultivated in a medium (10 mL, pH 6.0) containing glucose and/or odd chain fatty substrates, as the major carbon source, and 1% yeast extract in a 50 mL flask for 6–8 days at 28°C with reciprocal shaking (120 strokes/min).

Analysis of fatty acid composition and n-*pentadecane.* Extraction and determination of the mycelial fatty acids were described previously (11,12,14). *n*-Pentadecane was analyzed by gas liquid chromatography (GLC) in the same manner as for fatty acid methyl ester analysis except for the column temperature (140°C).

Lipid extraction and separation. Mycelia washed with water and *n*-hexane were treated twice with chloroform/methanol/water according to the procedure of Bligh and Dyer (18). The lipid extract was separated by thin-layer chromatography (TLC) on silica gel ($60F_{254}$, 200 × 200 × 0.25mm; E. Merck, Darmstadt, Germany). The spots were located under u.v. after spraying the plate with 0.2% dichlorofluorescein in ethanol. The solvent system used was petroleum ether/diethyl ether/acetic acid (82:18:1, v/v/v). The spots were directly scraped off for methanolysis.

Isolation of $C_{19:4}$ acid methyl ester from fungal mycelia and other methods. The procedures used for transmethylation and purification of $C_{19:4}$ acid were essentially the same as described previously (6). Measurements of mass and ^1H NMR (nuclear magnetic resonance) spectra, and other analyses were also carried out as described previously (6,11–14).

RESULTS

Formation of odd chain PUFAs from glucose. The mycelia of *Mortierella alpina* CBS 219.35 grown in a medium containing only glucose and yeast extract contained significant amounts of unusual fatty acids besides palmitic, stearic, oleic, linoleic, γ-linolenic, dihomo-γ-linolenic and arachidonic acids, which are commonly found in arachidonic acid-producing species of *Mortierella*. Two major components of these unusual fatty acids showed the same retention times as authentic $C_{15:0}$ and $C_{17:0}$ acid methyl esters, respectively, on both GLC and high performance liquid chromatography (HPLC). The others were also considered to be odd chain unsaturated fatty acids because of their retention times on GLC and HPLC, and the

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TABLE 1

Accumulation of Odd Chain PUFAs by Several Species of *Mortierella* in a Medium Containing Methyl Pentadecanoate or *n*-Pentadecane[a]

Strain	FAM[b] or NA[b] added (%)	Mycelial fatty acid composition[b] (%)												
		15:0	16:0	17:0	17:1	18:0	18:1	18:2	18:3	19:3	19:4	20:3	20:4	Others	Total odd chain FA
Subgenus *Mortierella*															
M. alpina 1S-4 AKU 3998	FAM 0.5	13.8	4.7	11.7	6.8	2.3	9.5	4.7	2.8	1.9	7.4	2.2	22.9	9.3	50.7
	NA 2.0	24.8	1.0	14.0	15.0	tr	5.0	3.1	2.8	1.8	11.6	tr	10.2	10.7	74.5
M. elongata 1S-5 AKU 3999	NA 2.0	47.4	1.7	9.5	12.3	tr	6.8	1.5	1.6	1.6	5.4	0.7	7.2	4.3	78.6
M. verticillata IFO 8575	NA 2.0	35.2	1.9	8.8	13.8	tr	7.2	5.3	2.3	1.0	3.7	tr	15.3	5.5	68.1
M. kuhlmanii CBS 157.71	NA 2.0	47.2	2.9	7.2	7.9	1.2	5.2	4.0	2.1	1.3	3.4	tr	8.2	9.4	75.6
M. alpina CBS 219.35	FAM 1.0	51.6	3.3	6.2	5.7	1.2	9.5	6.2	3.7	0.9	1.1	1.6	7.3	1.7	67.0
Subgenus *Micromucor*															
M. ramanniana	FAM 1.0	31.8	9.8	9.1	8.3	2.6	20.6	7.4	9.5	nd	nd	nd	nd	0.9	49.2
var. *angulispora* IFO 8187	NA 2.0	2.6	13.7	nd	nd	1.9	40.7	17.4	22.8	nd	nd	nd	nd	0.9	2.6
M. isabellina IFO 6739	FAM 1.0	28.1	10.1	2.5	2.7	1.8	29.1	13.7	11.7	nd	nd	nd	nd	0.3	33.3

[a]Each strain of *Mortierella* was grown in a medium containing 2% glucose, 1% yeast extract and methyl pentadecanoate or *n*-pentadecane, as indicated, pH 6.0, for 6–8 days at 28°C.

[b]Abbreviations used: FAM, methyl pentadecanoate; NA, *n*-pentadecane; 15:0, pentadecanoic acid; 16:0, palmitic acid; 17:0, heptadecanoic acid; 17:1, heptadecenoic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, γ-linolenic acid; 19:3, nonadecatrienoic acid; 19:4, nonadecatetraenoic acid; 20:3, dihomo-γ-linolenic acid; 20:4, arachidonic acid; FA, fatty acids; tr, trace; nd, not detectable.

TABLE 2

Comparison of Mycelial Fatty Acid Composition of *M. alpina* 1S-4 Grown With Odd Chain Fatty Compounds[a]

FAM or NA added[b]	Mycelial fatty acid composition[b] (%)												
	15:0	17:0	17:1	17:2	17:3 19:0	19:1	19:3	19:4	16:0	18:1	20:4	Others	Total odd chain FA
C15 FAM	13.8	11.7	6.8	tr	1.3	6.2	1.9	7.4	4.7	9.5	22.9	13.8	50.7
C19 FAM	tr	tr	tr	nd	20.9	nd	nd	nd	13.2	8.6	42.2	15.1	20.9
C11 NA	0.5	0.7	0.2	nd	0.3	nd	nd	nd	17.5	25.0	28.3	27.5	1.7
C13 NA	1.1	1.2	0.3	nd	0.2	nd	nd	nd	16.7	16.7	33.9	29.9	2.8
C15 NA	16.8	8.4	5.6	tr	0.8	tr	1.2	3.3	8.2	11.4	25.2	19.1	36.6
C17 NA	2.0	9.4	4.6	1.2	1.0	tr	0.7	0.8	16.6	23.6	18.5	21.6	19.7
C19 NA	0.8	2.8	1.2	tr	2.4	tr	0.3	0.4	16.0	26.4	20.1	29.6	9.6

[a]*M. alpina* 1S-4 was grown in a medium containing 2% glucose, 1% yeast extract and each of the indicated fatty acid methyl esters (0.5%) or *n*-alkanes (1.0%), pH 6.0, for 6–8 days at 28°C.

[b]Abbreviations used: C15 FAM, methyl pentadecanoate; C19 FAM, methyl nonadecanoate; C11 NA, *n*-undecane; C13 NA, *n*-tridecane; C15 NA, *n*-pentadecane; C17 NA, *n*-heptadecane; C19 NA, *n*-nonadecane; 17:2, heptadecadienoic acid; 17:3, heptadecatrienoic acid; 19:0, nonadecanoic acid; 19:1, nonadecenoic acid. For other abbreviations, see Table 1.

results described later regarding their GLC-mass and ^1H NMR spectra. These odd chain fatty acids accounted for 17.7% of the total mycelial fatty acids, and comprised $C_{15:0}$ (4.0%, by weight), $C_{17:0}$ (7.2), $C_{17:1}$ (5.2), $C_{19:3}$ (0.8) and $C_{19:4}$ (0.5) acids, whereas the odd chain fatty acids in mullet oil have been reported to account for about 25% of the total fatty acids, and to be comprised of $C_{15:0}$ (11.2%), $C_{17:0}$ (4.6), $C_{17:2}$ (2.5), $C_{19:4}$ (1.7) acids, plus others (1). Similarly, *M. alpina* var. *renispora* CBS 210.32, *M. hyalina* NRRL 6427 and *M. minutissima* IFO 8573 accumulated detectable amounts of C-17 and C-19 PUFAs.

Conversion of fatty substrates with an odd chain skeleton to odd chain PUFAs. To obtain greater amounts of odd chain PUFAs, several species of *Mortierella* were grown with *n*-pentadecane or methyl pentadecanoate, as shown in Table 1. All the arachidonic acid-producing species of *Mortierella*, which belong to the subgenus *Mortierella*, accmulated $C_{19:4}$ and $C_{19:3}$ acids together with C-15 and C-17 fatty acids, such as $C_{15:0}$, $C_{17:0}$ and $C_{17:1}$ acids. C-15 unsaturated fatty acids were not detected. On the other hand, species of *Mortierella* belonging to the subgenus *Micromucor*, which cannot produce C-20 PUFAs (10), accumulated C-17 PUFAs but not C-19 PUFAs. The total accumulation of odd chain fatty acids in arachidonic acid-producing species of *Mortierella* accounted for more than half of the total mycelial fatty acids. Both *n*-pentadecane and methyl pentadecanoate were efficiently converted to odd chain fatty acids, and exhibited essentially the same mycelial fatty acid profile. We selected *M. alpina* 1S-4 for the following experiments because of its high mycelial accumulation of $C_{19:4}$ acid.

M. alpina 1S-4 was grown with glucose and odd chain fatty acid methyl esters or odd chain *n*-alkanes, as shown in Table 2. C-15 and C-17 alkanes were efficiently converted to C-19 PUFAs. But fatty substrates with shorter and longer carbon chains (i.e., C-11, C-13 and C-19) were

TABLE 3

Production of Nonadecatetraenoic and Arachidonic Acids by *M. alpin* 1S-4 Grown with *n*-Pentadecane or *n*-Heptadecane[a]

	n-Pentadecane					*n*-Heptadecane				
		Mycelial PUFA content (mg/g dry mycelia)		PUFA yield (mg/mL)			Mycelial PUFA content (mg/g dry mycelia)		PUFA yield (mg/mL)	
(%)	Mycelial mass (mg/mL)	19:4[b]	Ara[c]	19:4	Ara	Mycelial mass (mg/mL)	19:4	Ara	19:4	Ara
3	14.8	31.5	8.7	0.47	0.13	14.3	27.4	5.9	0.39	0.08
4	13.0	24.1	5.3	0.31	0.07	14.7	37.8	5.0	0.55	0.07
5	13.0	19.8	4.4	0.26	0.06	15.4	44.4	5.9	0.68	0.09

[a] *M. alpina* 1S-4 was grown in a medium containing 3–5% *n*-pentadecane or *n*-heptadecane, as indicated, and 1% yeast extract, pH 6.0, for 7 days at 28°C.

[b] 19:4, 5,8,11,14-*cis*-Nonadecatetraenoic acid.

[c] Ara, arachidonic acid.

TABLE 4

Comparison of Mycelial Fatty Acid Composition of *M. alpina* 1S-4 Grown in the Presence or Absence of Sesamin[a]

Addition of sesamin	Mycelial fatty acid composition[b] (%)													
	15:0	16:0	17:0	17:1	18:0	17:2	18:1	18:2	18:3	19:3	19:4	20:3	20:4	Others
No	15.1	9.1	8.9	5.1	5.3	1.6	9.6	4.5	3.3	1.0	3.4	3.8	26.3	3.0
Yes	20.0	9.8	7.2	3.1	2.8	1.1	6.7	1.7	4.4	3.8	1.7	14.1	19.6	4.0

Addition of sesamin	19:3/19:4	20:3/20:4	Odd chain fatty acids (%)	Even chain fatty acids (%)	S+M[b] (%)	P[b] (%)
No	0.29	0.14	36.0	61.9	53.1	43.9
Yes	2.24	0.72	37.3	59.1	49.6	46.4

[a] *M. alpina* 1S-4 was grown in a medium containing 2% glucose, 2% *n*-pentadecane and 1% yeast extract supplemented with 0.01% of sesamin or unsupplemented, pH 6.0, for 6 days at 28°C.

[b] Abbreviations used: S, saturated fatty acids; M, monounsaturated fatty acids; P, fatty acids having more than two double bonds. For other abbreviations, see Tables 1 and 2.

not efficiently converted. When it was grown with C-15 or C-17 fatty substrates, the predominant odd chain fatty acids were $C_{15:0}$, $C_{17:0}$, $C_{17:1}$, $C_{17:2}$, $C_{17:3}$, $C_{19:3}$ and $C_{19:4}$ acids. C-15 unsaturated fatty acids and odd chain fatty acids of more than 21 carbons were not detected.

The data in Table 3 show that *M. alpina* 1S-4 could grow in a medium containing an odd chain *n*-alkane as a major carbon source and that the $C_{19:4}$ acid content reached 44.4 mg/g dry mycelia (0.68 mg/mL of culture broth) when it was grown in a medium containing 5% *n*-heptadecane and 1% yeast extract. The total odd chain fatty acids accumulated reached over 95% of the mycelial fatty acids. The $C_{19:4}$ acid accounted for 11.2%, whereas arachidonic acid accounted for only 1.5%.

Inhibitory effect of sesamin on desaturation of $C_{18:3}$ acid to $C_{19:4}$ acid. M. alpina 1S-4 was grown with *n*-pentadecane supplemented with 0.01% of (+)sesamin or unsupplemented, as shown in Table 4. (+)Sesamin and related lignan compounds are specific inhibitors of Δ5 desaturation (S. Shimizu *et al.*, unpublished data). Table 4 shows that the mycelial "19:3/19:4" ratio increased from 0.29 to 2.24 with the supplementation of sesamin. The mycelial "20:3/20:4" ratio increased from 0.14 to 0.72, without a change in the percentage of odd chain fatty acids (36% with no supplementation and 37% with supplementation) or that of PUFAs (44% with no supplementation and 46% with supplementation).

Distribution of odd chain PUFAs in extracted lipids. The lipids extracted from mycelia of *M. alpina* 1S-4 grown with 3% *n*-pentadecane (NA medium) or 2% glucose (G medium) were separated into the triacylglyceride (TG)-fraction (Rf=0.6), the polar lipid (PL)-fraction (Rf=0.02) and the *n*-alkane (NA)-fraction (Rf=0.85) (Table 5). The *n*-pentadecane remaining in the NA medium amounted to 6.1% of the supplemented *n*-pentadecane and mycelial *n*-pentadecane represented by the NA-fraction to 12.6%, so about 80% of the supplemented *n*-pentadecane was consumed during the cultivation and 12% of the consumed *n*-pentadecane was converted to mycelial fatty acids.

In the case of the NA medium, every odd chain fatty acid was found in both the TG- and PL-fractions, but the fatty acid compositions of these two fractions were different. Table 5 shows that the percentage of saturated

TABLE 5

Comparison of Mycelial Fatty Acid Composition of the TG[a]-Fraction and PL[a]-Fraction of *M. alpina* 1S-4 Grown in the Presence or Absence of *n*-Pentadecane[b]

Medium[c]	Fra[d]	FA (mg/g dry mycelia)	Fatty acid composition[d] (%)											
			15:0	16:0	17:0	17:1	18:1	17:3	18:3	19:3	19:4	20:3	20:4	Others
NA	TG	218.7	56.8	1.2	21.0	7.7	1.9	2.3	0.4	1.0	1.7	tr	0.4	5.6
NA	PL	23.9	15.4	2.4	7.5	19.7	12.5	3.4	8.7	2.1	14.0	0.5	3.9	9.9
G	TG	173.6	nd	18.2	nd	nd	17.5	nd	5.5	nd	nd	2.3	38.7	17.8
G	PL	27.5	nd	16.0	nd	nd	15.8	nd	5.7	nd	nd	2.5	42.4	17.6

[a] Abbreviations used: TG, triacylglyceride; PL, polar lipid.

[b] *M. alpina* 1S-4 was grown in the NA or G medium for 6 days at 28°C.

[c] The NA medium contained 3% *n*-pentadecane and 1% yeast extract, pH 6.0. The G medium contained 2% glucose and 1% yeast extract, pH 6.0.

[d] Fra, fraction. For other abbreviations used, see Tables 1 and 2.

odd chain fatty acids in the TG-fraction was high ($C_{15:0}$, 56.8%; and $C_{17:0}$, 21.0%) and the percentage of PUFAs was low ($C_{19:4}$, 1.7%; and $C_{20:4}$, 0.4%). On the other hand, the percentage of saturated odd chain fatty acids in the PL-fraction was relatively lower ($C_{15:0}$, 15.4%; and $C_{17:0}$, 7.5%) and that of PUFAs was higher ($C_{19:4}$, 14.0%; and $C_{20:4}$, 3.9%). In the case of the G medium, the fatty acid compositions of both fractions were almost the same, the $C_{20:4}$ acid content reaching about 40%. The ratio of the TG- and PL-fractions of the lipids from mycelia grown in the NA medium was 88:12, while that of the lipids from mycelia grown in the G medium was 86:14.

Identification of $C_{19:4}$ acid and the other odd chain PUFAs. $C_{19:4}$ acid methyl ester (13 mg) was isolated from the lipids extracted from 40 g of wet mycelia of *M. alpina* 1S-4 grown in a medium containing 4% *n*-pentadecane and 1% yeast extract, pH 6.0, for 8 days at 28°C as described previously (6). The mass spectrum of the isolated methyl ester showed peaks at *m/z* 304 (M^+; relative intensity, 2%), 250, 220, 180, 163, 150, 136, 119, 106, 93, 79 (base peak), 67, 55 and 41. The ^1H NMR spectrum in $CDCl_3$, with tetramethylsilane as an internal standard, showed signals at 0.90 (*t*, 3H, CH_3), 1.31 (*m*, 4H, CH_2), 1.68 (*m*, 2H, CH_2), 2.09 (*m*, 4H, CH_2), 2.30 (*t*, 2H, CH_2), 2.83 (*m*, 6H, CH_2), 3.63 (*s*, 3H, CH_3) and 5.38 ppm (*m*, 8H, C=C), which are the same as those in the case of authentic methyl arachidonate except that the peak at 1.31 ppm of methyl arachidonate, corresponding to methylene protons at the 17, 18 and 19 carbon atoms, indicates 6H. These data suggest that the structure of the isolated methyl ester was 5,8,11,14-*cis*-nonadecatetraenoic acid methyl ester. The other odd chain PUFA methyl esters were partly purified and determined by GLC-mass spectra to be as follows: nonadecatrienoic acid methyl ester, *m/z* 306 (M^+; relative intensity, 17%), 275, 222, 177, 163, 149, 136, 121, 107, 93, 79 (base peak), 67, 55 and 41; heptadecatrienoic acid methyl ester, *m/z* 278 (M^+, 13%), 194, 163, 149, 136, 120, 107, 93, 79, 67 (base peak), 55 and 41; heptadecadienoic acid methyl ester, *m/z* 280 (M^+, 22%), 249, 206, 164, 150, 136, 123, 109, 95, 81, 67 (base peak), 55 and 41.

DISCUSSION

Hoffmann and Rehm reported that three fungi belonging to th Mucorales, *Absidia spinosa, Cunninghamella echinulata* and *Mortierella isabellina*, which could not produce C-20 PUFAs, accumulated C-17 PUFAs but no C-19 PUFAs when grown with odd chain *n*-alkanes (4). Here we also show that *M. isabellina* and *M. ramanniana* var. *angulispora*, belonging to the subgenus *Micromucor*, which could not produce C-20 PUFAs, could accumulate some C-17 acids but no C-19 PUFAs. Some *Candida* yeasts, which are unable to form C-20 PUFAs, were also reported to produce C-17 acids but no C-19 PUFAs when grown on odd chain *n*-alkanes (5). These data indicate that only microorganisms that can produce C-20 PUFAs can produce C-19 PUFAs, and that microorganisms that are unable to produce C-20 PUFAs cannot produce C-19 PUFAs.

One of the predominant C-19 PUFAs produced by arachidonic acid-producing species of *Mortierella* was identified as 5,8,11,14-*cis*-nonadecatetraenoic acid, corresponding to arachidonic acid minus one carbon unit at the ω-terminal. The other odd chain PUFAs formed by these fungi were considered to be $C_{19:3}$, $C_{17:3}$ and $C_{17:2}$ acids, according to the mass spectra of their methyl esters. C-21 PUFAs have not been detected. These data suggest that the odd chain PUFAs were biosynthesized through the proposed pathway in Figure 1, which mimics the pathway for the biosynthesis of even chain PUFAs, the following successive reactions being involved: Oxidation of *n*-pentadecane to $C_{15:0}$ acid, elongation to $C_{17:0}$ acid, desaturation to 6,9,12-*cis*-$C_{17:3}$ acid *via* $C_{17:1}$ and $C_{17:2}$ acids as successive intermediates, elongation of $C_{17:3}$ acid to $C_{19:3}$ acid, and further desaturation of $C_{19:3}$ acid to $C_{19:4}$ acid. These odd chain PUFAs correspond to the equivalent even chain PUFAs minus one carbon unit at the ω-terminal. This is supported by the fact that only microorganisms that can produce C-20 PUFAs can produce C-19 PUFAs. The reports (2,3) that 9,12-$C_{17:2}$ and 6,9,12-$C_{17:3}$ acids were probably converted to 5,8,11,14-$C_{19:4}$ acid in the rat liver, and the results in Table 3 that show a significant decrease in the accumulation of arachidonic acid in *M. alpina* 1S-4 when a large amount of $C_{19:4}$ acid is produced also support this assumption.

We reported that (+)sesamin and some related lignans are specific inhibitors of the Δ5 desaturation reaction in *M. alpina* 1S-4 and rat liver (S. Shimizu, *et al.*, unpublished data). Mycelial dihomo-γ-linolenic acid, which is a substrate for Δ5 desaturation, increases with an accompanying decrease in mycelial arachidonic acid, a product

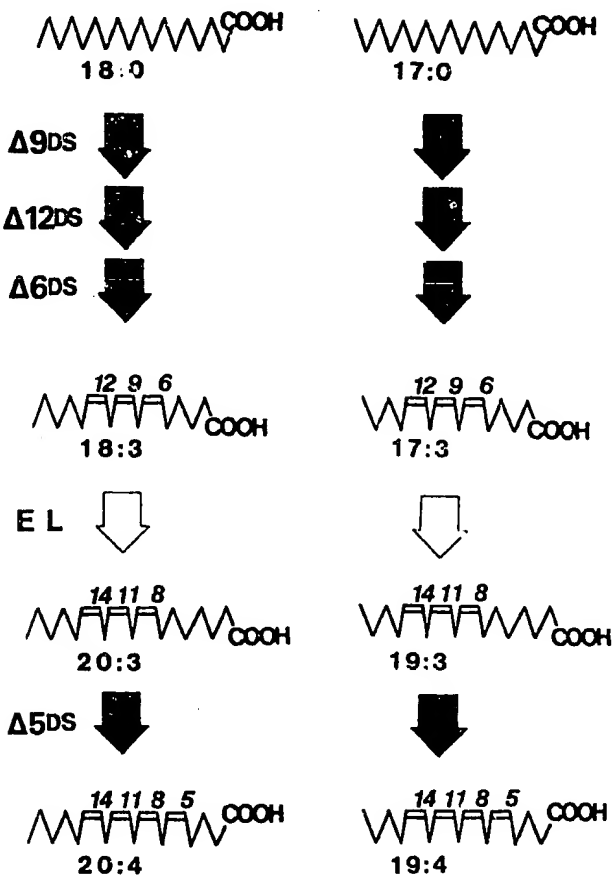

FIG. 1. Proposed pathway for the biosynthesis of odd chain PUFAs in *Mortierella*. Abbreviations used: DS, desaturation; EL, elongation. For other abbreviations, see Tables 1 and 2.

of Δ5 desaturation. The composition of the other mycelial fatty acids does not change when the fungus is grown with (+)sesamin. Here we show that mycelial $C_{19:3}$ acid also increased with an accompanying decrease in $C_{19:4}$ acid when *M. alpina* 1S-4 was grown with *n*-pentadecane in the presence of (+)sesamin. This phenomenon suggests that both the desaturation of $C_{19:3}$ acid to $C_{19:4}$ acid and that of dihomo-γ-linolenic acid to arachidonic acid are catalyzed by the same enzyme. This also supports the above assumption that the biosynthesis of odd chain PUFAs mimics that of even chain PUFAs.

About 90% of the supplemented *n*-pentadecane was incorporated into the mycelia of *M. alpina* 1S-4, and about 80% of that was consumed by the fungus. Twelve percent of the consumed *n*-pentadecane was converted and accumulated as mycelial fatty acids, but the rest of the consumed *n*-pentadecane is considered to have also been converted to pentadecanoic acid, which is then metabolized to various cellular components. This seems to be reflected by the fact that the percentage of $C_{15:0}$ acid in the TG-fraction derived from the mycelia grown in the NA medium was very high (56.8%) (Table 5). The PUFA contents then were low ($C_{19:4}$, 1.7%; and $C_{20:4}$, 0.4%). On the other hand, the PL-fraction of the same mycelia contained relatively lower percentages of $C_{15:0}$ (15.4%) and $C_{17:0}$ (7.5%) acids and higher percentage of $C_{19:4}$ (14.0%) and $C_{20:4}$ (3.9%). Since all odd chain fatty acids were found not only in the TG-fraction but also in the PL-fraction, they are considered to occur and function as constituents in biomembranes. The higher percentages of PUFAs such as $C_{19:4}$ and $C_{20:4}$ acids in the PL-fraction show that these PUFAs are necessary for the functions of a biomembrane and are probably selectively incorporated into the biomembrane.

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Arachidonic Acid Production by Fungi

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After preliminary screening, *Mortierella alpina* and *Mortierella elongata* were compared with respect to arachidonic acid content. *M. alpina* ATCC 16266 produced 2.1 g of arachidonic acid per liter in media containing 10% glucose while the highest percentage of arachidonic acid in lipid (43.3%) was observed at a glucose concentration of 2%. Arachidonic acid content in lipids increased to 66% during storage.

Arachidonic acid is a precursor of numerous eicosanoids and other compounds which are presently the subject of extensive medical research (6, 14, 19). Although arachidonic acid is presently isolated from animal adrenal gland and liver and from sardines, the yield is only 0.2% (wt/wt) (1). Arachidonic acid is also found in the cells of ciliated protozoa, amoebae, algae, and other microorganisms (3, 5, 8, 22) including *Mortierella* species (15, 17, 20, 21).

Media and culture conditions. GY medium contained the following (in grams per liter): glucose, 20; and yeast extract, 10. YM medium (20) consisted of the following (in grams per liter): glucose, 10; polypeptone, 5; yeast extract, 3; and malt extract, 3. HD medium (10) contained the following (per liter): glucose, 30.0 g; yeast extract, 5.0 g; KH_2PO_4, 2.4 g; KNO_3, 1.0 g; $CaCl_2 \cdot 2H_2O$, 0.1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $FeCl_3 \cdot 6H_2O$, 15 mg; $ZnSO_4 \cdot 7H_2O$, 7.5 mg; and $CuSO_4 \cdot 5H_2O$, 0.5 mg. Fungal strains were maintained on GY medium supplemented with 3% agar and were transferred every 2 months. Cultures were first grown on HD medium for 48 h at 25°C. This culture (5% [vol/vol]) was used to inoculate the production medium. Inoculum and production cultures were prepared in 250-ml Erlenmeyer flasks containing 50 ml of medium and shaken at 300 rpm on an orbital shaker.

Lipid analysis. Lipids were extracted from the dried fungal biomass by the method of Bligh and Dyer (4), and the extract was dried at 36°C and methylated (11). The fatty acid methyl esters, dissolved in *n*-hexane, were analyzed by gas chromatography. The degree of unsaturation in the lipid fraction was calculated by the method of Kates and Baxter (12).

Screening at 25°C. Although several fungal strains have been screened for lipid accumulation, only selected strains which have some potential for intracellular lipid production are reported here. *Mortierella alpina* ATCC 32221 did not produce arachidonic acid at 25°C (Table 1). *Conidiobolus* species exhibited low arachidonic acid content. Arachidonic acid content of the biomass of the remaining strains ranged from 2.2 to 8.4% (wt/wt). The lowest levels of biomass production and arachidonic acid yield occurred in cultures grown in YM medium for all strains. Of the three *Mortierella elongata* strains tested, NRRL 5513 produced the highest biomass and the highest arachidonic acid yield in GY and HD media. In GY medium, NRRL 5513 had a low total lipid content (11.5%) but arachidonic acid accounted for 33.9% of this lipid. In contrast, in NRRL 5513 grown in HD medium, the total lipid content was higher but the percentage of arachidonic acid in lipid was lower. Arachidonic acid yield from *M. elongata* NRRL 5513 grown in HD medium was 22% higher than the corresponding value for NRRL 5513 grown in GY medium.

M. alpina ATCC 16266 and ATCC 42430 were the best producers of arachidonic acid of this species. The highest arachidonic acid yields were observed in strains grown in HD medium, where the arachidonic acid content in lipid was 26.4 to 26.9%. In GY medium, arachidonic acid yield was lower, although it accounted for 41.6 to 43.3% of total lipid. Contents of arachidonic, oleic, linoleic, and linolenic acids in strain ATCC 16266 were 43.3, 13.4, 8.5, and 1.5% (wt/wt) of total lipid, respectively. Corresponding values for strain ATCC 36965 were 3.9, 25.2, 40.0, and 6.3% (wt/wt) total lipid.

Screening at 11°C. *Mortierella* strains were also grown at 11°C in HD medium for 10 days (Table 2). For *M. elongata*, biomass values were higher and arachidonic acid contents of biomass were lower when strains were grown at 11 than at 25°C. While the average arachidonic acid yields of the three strains grown at 11°C (0.39 g/liter) and those for the same strains grown at 25°C (0.38 g/liter) were similar, productivities (arachidonic acid produced per liter per day) were much lower for strains grown at 11 than at 25°C. Although biomass values observed for *M. alpina* ATCC 16266 and ATCC 42430 were higher when the strains were grown at 11°C, lipid and arachidonic acid contents of biomass were significantly lower, and, overall, higher yields were observed at a growth temperature of 25°C. Traces of arachidonic acid were synthesized by *M. alpina* ATCC 32221 at 11°C, although no such production was observed at 25°C.

M. alpina ATCC 16266, which manifested the highest yields of arachidonic acid, was selected for further studies. Although strains grown on GY medium produced a lower overall yield than those grown on HD medium, GY medium was selected for further investigation because strains grown on it produced a higher content of arachidonic acid in lipid (43%) than did strains grown on HD medium (27%).

Effect of initial pH. When the initial pH of the medium was varied, strain ATCC 16266 grew well in the pH range of 3.8 to 8.0 (Fig. 1). Lipid content of biomass, degree of lipid unsaturation, and arachidonic acid yield of ATCC 16266 were also highest when the initial pH was 6.0.

Effect of carbon source. When different compounds were tested as carbon source in GY medium, growth of ATCC 16266 was found to be very poor with lactose, starch, and sucrose as carbon source, moderate with maltose, fructose, and glucose, and very good with linseed oil and glycerol (Table 3). The arachidonic acid content in lipids was above

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TABLE 1. Comparison of fungal strains with respect to growth, lipid content, and arachidonic acid production in selected media[a]

Strain	Medium	Biomass (g/liter)	Lipids in biomass (% [wt/wt])	Arachidonic acid		
				In biomass (% [wt/wt])	In lipids (% [wt/wt])	Yield (g/liter of broth)
Mortierella elongata						
ATCC 16271	GY	7.6	21.5	3.2	15.0	0.25
	YM	6.6	21.3	3.3	15.5	0.22
	HD	11.1	27.0	2.6	9.5	0.29
ATCC 24129	GY	7.8	20.4	4.7	23.0	0.37
	YM	6.2	12.9	2.2	17.2	0.14
	HD	9.0	16.0	3.2	19.8	0.29
NRRL 5513	GY	12.7	11.5	3.9	33.9	0.49
	YM	8.7	[illegible]	2.3	22.2	0.19
	HD	13.7	22.3	4.2	19.0	0.58
Mortierella alpina						
ATCC 16266	GY	12.1	13.2	5.7	43.3	0.69
	YM	8.9	12.5	3.4	27.1	0.30
	HD	13.0	31.2	8.4	26.9	1.09
ATCC 42430	GY	11.7	10.0	4.2	41.6	0.49
	YM	8.6	11.4	4.8	42.4	0.41
	HD	12.4	23.0	6.1	26.4	0.75
ATCC 32221	GY	10.8	1.6	0.0	0.0	0.0
	YM	7.8	1.6	0.0	0.0	0.0
	HD	13.7	1.1	0.0	0.0	0.0
ATCC 36965	GY	10.5	3.3	0.1	3.9	0.01
Conidiobulus obscurus						
ATCC 36369	GY	2.9	1.0	0.1	10.0	0.0
ATCC 42977	GY	2.1	1.3	0.1	9.5	0.0

[a] Culture conditions: 25°C, 6 days. Data are the averages of three replicates.

40% (wt/wt) with starch, maltose, glucose, and fructose used as carbon source. Although glycerol utilization produced the highest yield of arachidonic acid, the arachidonic acid content in lipids was quite low. The highest arachidonic acid yields were produced on GY medium with glucose as the carbon source. The effect of glucose concentration on arachidonic acid production was also investigated (Fig. 2). Arachidonic acid contents in lipids, in biomass, and per liter of culture were at their maximum in medium with glucose concentrations of 20, 50, and 100 g/liters, respectively.

Effect of nitrogen source. Of various nitrogen sources added separately at a 1% concentration to the medium containing 10% glucose, yeast extract followed by peptone resulted in the highest arachidonic acid yields of 2.09 and 0.74 g/liter, respectively. Growth was very poor with other nitrogen sources. High percentages of arachidonic acid in

TABLE 2. Comparison of *Mortierella* strains with respect to growth, lipid content, and arachidonic acid production in HD medium at 11°C[a]

Strain	Biomass (g/liter)	Lipids in biomass (% [wt/wt])	Arachidonic acid		
			In biomass (% [wt/wt])	In lipids (% [wt/wt])	Yield (g/liter of broth)
M. alpina					
ATCC 16266	17.8	22.6	5.0	22.2	0.90
ATCC 32221	4.2	1.6	0.1	7.1	0.01
ATCC 42430	21.7	10.7	3.4	32.1	0.74
M. elongata					
ATCC 16271	15.8	27.7	2.5	9.0	0.39
ATCC 21429	13.0	21.1	2.7	12.8	0.35
NRRL 5513	14.6	23.3	3.0	13.0	0.44

[a] Data are the averages of three replicates.

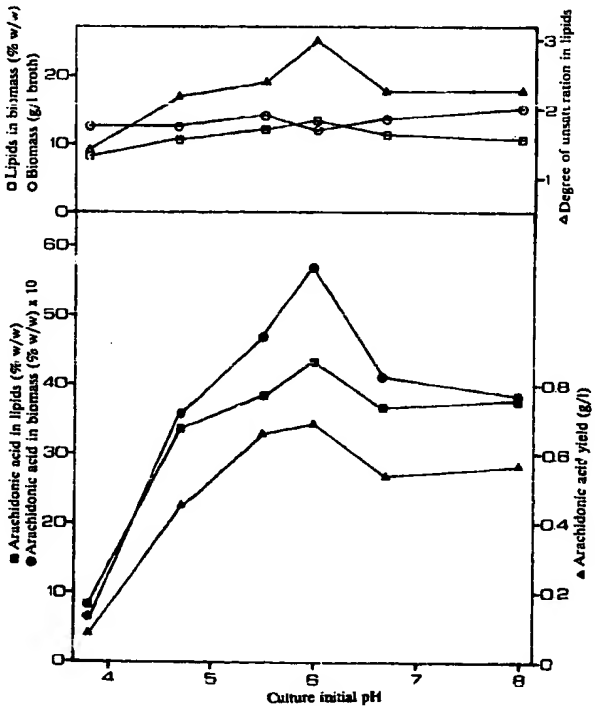

FIG. 1. Effect of initial culture pH on growth of strain and on arachidonic acid and lipid produced by *M. alpina* ATCC 16266.

TABLE 3. Effect of carbon source on growth, lipid content, and arachidonic acid production by *M. alpina* ATCC 16266[a]

Carbon source	Biomass (g/liter)	Lipids in biomass (% [wt/wt])	Degree of unsaturation in lipids	Arachidonic acid		
				in biomass (% [wt/wt])	in lipid (% [wt/wt])	Yield (g/liter of broth)
Fructose	12.8	12.2	2.43	4.9	40.1	0.63
Glucose	12.1	13.2	2.99	5.7	43.3	0.69
Glycerol	15.6	20.5	2.06	5.8	28.4	0.91
Lactose	4.0	5.1	2.23	1.7	33.6	0.07
Linseed oil	24.4	51.2	2.20	0.9	1.8	0.22
Maltose	13.5	11.3	2.45	4.8	42.2	0.64
Starch	4.6	5.0	2.48	2.0	40.3	0.09
Sucrose	3.8	4.4	2.10	1.2	27.9	0.05

[a] Culture conditions consisted of GY medium containing 2% (wt/vol) of each carbon source incubated at 25°C for 6 days. Data are the average of three replicates.

the lipid fraction were observed with urea (25.1%) and malt extract (23.8%) used as the nitrogen source.

Aging of mycelium. When the biomass, obtained with 5% glucose medium, was stored at 22°C for 1 week, the arachidonic acid content increased from 8.3 to 13.5% of dry biomass and from 25.3 to 41.3% of the lipid fraction. Similarly, arachidonic acid content in mycelium obtained with 2% glucose medium also increased from 5.7 to 8.7% dry weight and from 43.3 to 65.9% in the lipid fraction.

We have identified a strain of *M. alpina* (ATCC 16266) and media conditions which resulted in the production of 1.90 to 2.09 g of arachidonic acid per liter, representing 6.8 to 8.3% of biomass dry weight. The arachidonic acid yield observed is the highest so far reported in fungal shake-flask cultures (15, 21). Significantly higher arachidonic acid contents in biomass may be achieved by growth on agar plates (20). When recovered mycelium was stored for 7 days, arachidonic acid content increased to 65.9% of total lipid. A similar increase in arachidonic acid content to 67.4% was also reported by Shinmen et al. (17). With many microorganisms, a decrease in unsaturated fatty acid content occurs on aging (7). However, polyunsaturated fatty acids in *Ochromonas danica* (9) and *Phaeodactylum tricornutum* (2) increased with aging. Maximum yields of arachidonic acid in cultures of *M. elongata* were 0.96 to 0.99 g/liter (15, 21). In their fermentor studies, Shinmen et al. (18) reported a maximum arachidonic acid yield and percentage of arachidonic acid in lipid of 3.6 g/liter and 35% (wt/wt), respectively.

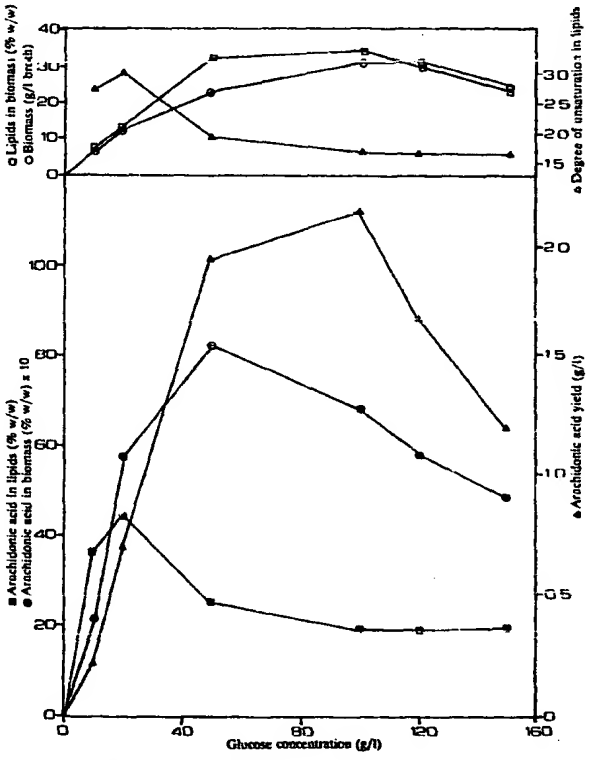

FIG. 2. Effect of glucose concentration on growth of strain and on arachidonic acid and lipid produced by *M. alpina* ATCC 16266.

Yields of arachidonic acid for *M. elongata* and *M. alpina* strains, cultured at 11°C, were similar to yields observed at 25°C in the same medium. In contrast, the production of eicosapentaenoic acid by several *Mortierella* species (18) and by *Chlorella minutissima* (16) is stimulated at reduced temperatures. Glucose was also reported to best support cell growth and arachidonic acid production by *M. elongata*, maximum arachidonic acid production being observed in media containing 100 g of glucose per liter (21). The accumulation of oleic and linoleic acids by the low-arachidonic-acid-producing strain, *M. alpina* ATCC 36965, suggests that the enzymatic desaturation and/or elongation reactions for conversion of linoleic to arachidonic acid (13) do not operate efficiently in this strain.

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